



**QUEEN'S
UNIVERSITY
BELFAST**

The crustacean cuticle does not record chronological age: New evidence from the gastric mill ossicles

Becker, C., Dick, J. T. A., Cunningham, E. M., Schmitt, C., & Sigwart, J. D. (2018). The crustacean cuticle does not record chronological age: New evidence from the gastric mill ossicles. *Arthropod structure & development*, 47(5), 498-512. <https://doi.org/10.1016/j.asd.2018.07.002>

Published in:

Arthropod structure & development

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

Copyright 2018 Elsevier Ltd.

This manuscript is distributed under a Creative Commons Attribution-NonCommercial-NoDerivs License

(<https://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits distribution and reproduction for non-commercial purposes, provided the author and source are cited.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access

This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: <http://go.qub.ac.uk/oa-feedback>



**QUEEN'S
UNIVERSITY
BELFAST**

The crustacean cuticle does not record chronological age: New evidence from the gastric mill ossicles

Becker, C., Dick, J. T. A., Cunningham, E. M., Schmitt, C., & Sigwart, J. D. (2018). The crustacean cuticle does not record chronological age: New evidence from the gastric mill ossicles. *Arthropod structure & development*, 47(5), 498-512. <https://doi.org/10.1016/j.asd.2018.07.002>

Published in:
Arthropod structure & development

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

Copyright 2018 Elsevier Ltd.
This manuscript is distributed under a Creative Commons Attribution-NonCommercial-NoDerivs License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits distribution and reproduction for non-commercial purposes, provided the author and source are cited.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.



This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No H2020-MSCA-IF-2014-655661.

This copy of the accepted manuscript is provided to enable dissemination through Open Access to the scientific data; the version of record is that provided by the publishers.

The crustacean cuticle does not record chronological age:

New evidence from the gastric mill ossicles

Carola Becker^{1,2,*}, Jaimie T. A. Dick^{1,3}, Eoghan Mánus Cunningham¹, Clemens Schmitt⁴, Julia D. Sigwart¹

¹Queen's University Marine Laboratory, 12-13 The Strand, Portaferry BT22 1PF, Northern Ireland

²Humboldt Universität zu Berlin, Department of Biology, Comparative Zoology, Philippstr. 13, Haus 2, 10115 Berlin, Germany (present address)

³Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, MBC, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

⁴Max Planck Institute of Colloids and Interfaces, Potsdam-Golm Science Park, Am Mühlenberg 1 OT, Golm, Germany

* Corresponding author: Carola Becker, carolabecker.germany@gmail.com

Co-authors email addresses: j.dick@qub.ac.uk, ecunningham18@qub.ac.uk, clemens.schmitt@mpikg.mpg.de, j.sigwart@qub.ac.uk

Abstract

A proposed method to determine chronological age of crustaceans uses putative annual bands in 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 of age. However, recent studies presented conflicting findings on the dynamics of gastric mill ossicles during ecdysis. We herein study cuticle bands in ossicles in four species of commercially important decapod crustaceans (*Homarus gammarus*, *Nephrops norvegicus*, *Cancer pagurus* and *Necora puber*) in different phases of the moult cycle using dissections, light microscopy, micro-computed tomography and cryo-scanning electron microscopy. Our results demonstrate that the 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 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 cuticle structures where 'annual growth bands' have been reported should be investigated urgently. Based on our results, there is no evidence for a causative link between cuticle bands and annual moult increments, meaning it is unreliable for determining crustacean age.

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

1. Introduction

A record of the age of individual organisms can manifest as annual rings or bands in hard skeletal 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 of past and future environmental impacts on tree growth (Creber, 1977). Similarly, age-registering hard structures are used in a variety of vertebrate and invertebrate groups. In fish, otoliths or scales can record annual growth rings (Campana, 2001); in bivalves, shells possess annual bands (Abele et al., 2009); and the statoliths of squid can show rings which have been 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, 1973; Hartnoll, 2001; Vogt, 2012).

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 commonly based on size modal analysis or on the observation of intermoult duration and increase in size at moulting, from specimens in captivity or tagging in wild populations (Vogt, 2012; Wahle & Fogarty, 2006). This method requires well constrained seasonality of reproductive cycles or 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 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 chronological age, since its deposition rate is influenced by metabolic and environmental conditions (O'Donovan and Tully, 1996). The lipofuscin approach therefore requires calibration for each species and different environmental conditions by using specimens of known age (Maxwell et al., 2007). Direct observations of crustacean ageing and longevity in the field are only possible through mark-release-recapture approaches, which can be very cost-intensive and time consuming (see Vogt, 2012 for a comprehensive review on ageing crustaceans).

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 retained through the moult. Evidence for such retention was reported in the original publications, as: (1) the absence of certain gastric mill ossicles in exuviae; and (2) the perpetuation of a calcium-binding fluorescent live marker in the endocuticle of ossicles through several moults (calcein 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 white-clawed crayfish, *Austropotamobius pallipes* (Sheridan et al., 2016) and the shore crab *Carcinus maeans* (Sheridan & O'Connor, 2018) and instead the ossicles were shed into the stomach 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).

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

(insert table 1)

1.3 The crustacean cuticle and the moult

Prior research on crustacean moulting has mostly focussed on the cuticle of the carapace and limbs (Welinder, 1974; 1975a, b). However, the cuticular exoskeleton is a continuous sheet that not only forms the outer shell but also lines several internal organs, including the structures in the foregut, and develops skeletal elements inside the body (Davie et al., 2008). The crustacean cuticle is always organised in distinct layers, which are characterised by their location, chemical 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 epicuticle. The outermost epicuticle is a generally thin and waxy layer which can also form scales, setae and teeth (as in the zygocardiac and urocardiac ossicle, see Fig. 3c). The procuticle constitutes the main component of the exoskeleton and is divided into a proximal endocuticle and a distal exocuticle. The membranous layer is per definition the unmineralised, most basal part of the endocuticle and directly overlies the hypodermis (Fabritius et al., 2016). In the procuticle, the protein-chitin fibers or fibrils are arranged in horizontal planes which are stacked helicoidal and form a twisted plywood structure (Davie et al., 2015; Fabritius et al., 2016; see also Fig. 2b). Endo- and exocuticle therefore appear multi-lamellate. These lamellae do not correspond to the light 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).

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

(Carmichael, 2013). All those parameters form the basis for stock assessment and the sustainable management of fisheries species. It is, however, absolutely critical that evidence-based fisheries management rests upon accurate interpretation of data. In the present study, we applied different 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* (European Lobster), *Nephrops norvegicus* (Norway Lobster), *Cancer pagurus* (Brown Crab) and *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 250 μm . Additionally, synchrotron micro-computed tomography and cryo-scanning electron microscopy was used to study the nature of cuticle bands in intermoult specimens. We then tested 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 moulted specimens using stereo microscopy, light microscopy and micro-computed tomography.

2. Methods

2.1 Specimen sampling

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 (54°23'.45N 05°37'.45W) and centrally in the lough (Tip Reef, 54°27'.55N 05°34'.80W). Additionally, freshly moulted specimens of *H. gammarus* and *C. pagurus* were obtained directly 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*: 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 on flow-through seawater until sacrificed. Live intermoult specimens were individually labelled and measured (carapace length, CL and carapace width, CW). Specimens were maintained in large outdoor tanks with a sea water flow through system and air supply at Queen's Marine Laboratory in Portaferry, UK (QML). Each species was kept in groups of five to 20 specimens per tank. Animals were fed *ad libitum* using locally sourced fish and mussels. Pipe sections of different sizes were provided as shelter for *H. gammarus* and *N. norvegicus*. Outdoor tanks were covered for shade and to exclude potential predators.

Specimens were monitored frequently to recover freshly moulted specimens together with their shed 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.

2.2 Dissections and sample preparation

In total, two specimens of *H. gammarus*, seven of *N. norvegicus*, three of *Necora puber*, and six 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 mills were immersed in a solution of 10% potassium hydroxide in distilled water and boiled for 10-25 minutes to remove soft tissues to improve observations. Gastric mills were then opened through a longitudinal incision on the ventral face to expose the zygo-cardiac, uro-cardiac, meso-cardiac and ptero-cardiac ossicles. The stomach content of freshly moulted specimens was searched for ossicles. The gastric mills in exuviae were soft and collapsed and therefore immersed in distilled water to facilitate dissections and observation. Samples were studied and photographed using an Olympus SZX16 dissection microscope equipped with an Olympus E600 camera. Images were processed in Adobe Photoshop version CS5.1 and assembled to figure plates.

2.3 Microscopic visualisation

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 for 10-25 minutes to remove soft tissues before gastric mills were dissected and ossicles extracted. Ossicles were subsequently air-dried, embedded in epoxy resin (Logitech Epo-Flo type 301) and hardened overnight at 40°C. Sample blocks were then cut with a Logitech GTS10 diamond saw and processed in a diamond lab. Blocks were polished using silicate carbide grinding paper at grade 320 and loose silicon carbide powder at grade 600 on a glass plate. The processed 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 WG2 Logitech polishing unit and studied and photographed 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).

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.

The thorax of this sample was then dehydrated through an ascending series of ethanol solutions and infiltrated (Shandon Hypercenter XP, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and embedded for transverse sections through the gastric mill in a paraffin block. Sections 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 “light green” stain (MORPHISTO®, Germany). Covered slides were studied and photographed 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).

The thorax of a specimen of *N. norvegicus* (see Table 2) was prepared for tomography by dehydration through an ascending ethanol series and chemically dried using hexamethyldisilazane (HMDS). The micro-computed tomography scan was conducted at the Museum für Naturkunde Berlin (Germany) using a Phoenix nanotom X-ray|s tube at 100 kV and 150 μA , generating 2000 projections. The effective voxel size was 20 μm , the detector timing 750 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 produced and exported with software VGStudio Max (Volume Graphics, Heidelberg). Image stacks were processed for volume renderings and surface reconstructions with software Amira version 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 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>).

Scanning electron micrographs were obtained in cryo conditions with a Cryo SEM (Jeol JSM7500F) 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 (-130°C), and cracked open with a scalpel blade. Sublimation was performed heating the sample preparation chamber from -130°C to -95°C (which took approximately 5 minutes) to remove 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 SEM stage at -130°C and a accelerating voltage of 3kV.

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).

3.1 Cuticle structure

Our data on the fate and growth dynamics of gastric mill ossicles includes two major lines of evidence. First, a combination of microscopic visualisation techniques allowed us to infer the internal construction of previously described bands within the cuticle. Sections of gastric mill ossicles showed cuticle bands (Fig. 1). However, the readability of sections cut at $\sim 200\ \mu\text{m}$ varied. In images obtained from synchrotron micro-computed tomography at three-dimensional resolutions of $\sim 8\ \mu\text{m}$, internal bands of differential density were visible (Fig. 2a). In cryo-scanning 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 indicates that the variation in material density within the cuticle is not readily visible from surface structure (SEM), but it is only observable when viewed by x-ray or transmitted light.

3.2 Moulting experiments

The second major line of evidence comprises data from whole animals observed through the 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 ossicles are calcified, which is visually apparent from their white to creamy colour and opaqueness (Figs. 3b, c, 4b, 7b). In freshly moulted specimens, the gastric teeth are present but the ossicles remain incomplete, they lack calcification and therefore appear translucent (Figs. 4c, 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 in the stomach of one freshly moulted specimen of *H. gammarus* and five specimens of *N. 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 material and different sets of ossicles. Due to the moult occurring during the night, the exact delay 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 pterocardiac 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.

Gastrolith material (Fig. 4d, 5) and zygocardiac, pterocardiac and mesocardiac ossicles (Figs. 4e, 5) were present in the stomach of five specimens of *N. norvegicus* recovered the day after an overnight moult (maximum up to 18 hours postmoult). In specimens that were recovered later 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 of *N. norvegicus* generally resumed feeding, and in five specimens sacrificed at this stage the stomach was filled with crushed food remains rather than gastrolith material (not listed in suppl. 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 only slightly soft. The three specimens received from fishermen were however extremely soft, but the exact time elapsed since moulting was unknown as the moult occurred in a deployed creel. In contrast to the other species, in *C. pagurus*, the stomach of the three freshly moulted specimens was found to be empty. Even in extremely soft, jelly-like specimens of freshly moulted *C. pagurus*, ossicles were not present in the gastric mill, nor were any food remains apparent. However, in recently moulted specimens of *C. pagurus* (as in all studied species), the gastric mill ossicles were completely decalcified and translucent (compare Fig. 7b and c). The same uncalcified gastric ossicle precursors were observed in *H. gammarus*, *N. norvegicus* and *N. puber* (Figs. 4c, 6d), and any observed variation in colour or texture is likely to be related to the unquantified time elapse between actual moult and preservation of specimens.

3.3 Light microscopic observations

Histological sections of a premoult specimen of *N. norvegicus* allowed the observation and study of the old cuticle that is going to be shed at the imminent moult and the new cuticle which is forming underneath simultaneously (Fig. 8). The zygocardiac ossicles were easily identified in transverse sections of the gastric mill by their paired structure and the gastric teeth which are characterised by a thick red-staining epicuticle that impregnates the tooth (Fig. 8a-d). Sections of the zygocardiac ossicle in the region of the tooth show the thick old cuticle (Fig. 8a, b) already delaminated from the gastric tooth. All cuticle layers (endo-, exo-, and epicuticle) are present in 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 incomplete and is composed only of the thick red-staining epicuticle and a thin underlying 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 bands, is structurally different from the gastric tooth by having a very thin epicuticle (Fig. 8e, f); however, the signs of an imminent moult are the same as in the tooth: all layers of the old cuticle, also the endocuticle which contains light and dark bands, are delaminated from the underlying hypodermis which is secreting the new cuticle (Fig. 8f).

4. Discussion

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.

4.2 The cuticle during the moult cycle

The moult cycle of crustaceans can be divided into different stages (Roer and Dillaman, 1984). The intermoult is the hard-shelled equilibrium condition in which a crustacean spends most of its 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 formation of the new epi- and endocuticle take place in the late premoult, thus the next skeleton 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 endocuticle and the mineralisation of the procuticle occurs gradually after moulting; it can take several days to complete this process before a specimen has fully hardened (Roer and Dillaman, 1984).

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 musculature attaches. Several ossicles carry teeth and form a chewing apparatus, the gastric mill (Maynard and Dando, 1974; Figs. 3-7). The epicuticle of the teeth is impregnated with silica, which gives them an amber-coloured appearance (Vatcher et al., 2015; Figs. 3c, 5b, c, 7b). The largest and most dominant structures of the dorsal ossicle complex within the gastric mill have been used as putative ageing structures (see Table 1). Importantly, these structures are still connected 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.

The structures within the gastric mill are complex and the individual ossicles are often challenging 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 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 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, for example, the mesocardiac ossicle differs remarkably between the crab and lobster species we

herein studied. The mesocardiac ossicle of *H. gammarus* and *N. norvegicus* is wide, strongly calcified and well separated from the adjoining urocardiac ossicle (see Fig. 3b). The same structure is narrow and fused with the urocardiac ossicle in *N. puber* and *C. pagurus* (see Fig. 7b). 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 diverse that it has frequently been used in phylogenetic reconstructions (Brösing, 2008; Brösing 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 al., 2015a, b). The fate of gastric mill ossicles during ecdysis has previously only been studied by Vatcher et al. (2015) and Sheridan et al. (2016). Vatcher et al. (2015) studied the mesocardiac and urocardiac ossicle in *C. sapidus* using light microscopy and elemental mapping, with special regard to the mineralisation of those structures during the moult cycle. Sheridan et al. (2016) looked at the fate of gastric mill ossicles in *N. norvegicus* from a macroscopic perspective. These studies also found that gastric mill ossicles were lost during the moult.

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 varying ossicles (Brösing, 2002), such that identification and classification of individual component structures can be very challenging. Different species examined herein showed 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 together with the paired zygocardiac and pterocardiac ossicles. In *N. puber*, only the zygo- and pterocardiac ossicles were present while the mesocardiac ossicle was not found. The mesocardiac ossicle in crabs is smaller and less calcified than the homologous structure in lobsters; this relative fragility might explain why it was not recovered from the stomach in *N. puber*. In *H. gammarus*, an 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 during the moult and because most of them are much smaller than the prominent teeth-bearing ossicles mentioned above.

The only species in which we were not able to find any ossicles in the stomach content of freshly moulted specimens was *C. pagurus*. However, the exact timing of the moult was not directly established for these individuals. In contrast to our findings for the other study species, in which the stomach was either filled with ossicles or food remains, the stomachs of *C. pagurus* were completely empty. This may suggest that resorption and remobilisation of minerals may occur differently in *C. pagurus* than in the other species, but importantly the ossicles were definitively not retained in the skeleton. We also investigated the gastric mill in exuvia of the studied species, 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 by Brösing (2014), who investigated the foregut in exuviae of several decapod species (*C. pagurus*, *Maja crispata* and *Pseudosesarma moeschi*) and found that ossicles of the gastric mill were incomplete, degraded and decalcified. The gastric teeth, however, were present and complete in the exuviae of those species. The observation of incomplete gastric mills in exuviae may explain why earlier studies concluded that these internalized structures are not shed but retained through moulting. Kilada et al. (2012) correctly described that the mesocardiac ossicle was missing in exuviae of *H. americanus*, and the swamp crayfish, *Procambarus clarkii*. However, their conclusion that the mesocardiac ossicle must be retained in these two species is neither supported by our 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 moulting on the gastric mill would be variable. We found it challenging to control the timing and 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 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 the moult.

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 experiments, and the anatomical explanation for their observations. Specimens of *H. americanus* (Kilada et al., 2012) and the red claw crayfish *Cherax quadricarinatus* (Leland et al., 2015) were dyed with the fluorescent marker calcein that binds to calcium. The live specimens were then allowed to moult several times before they were sacrificed. In both studies, the calcein mark was still present in the cuticle after the moult. This was interpreted as evidence for trans-moult retention of the endocuticle (Kilada et al., 2012; Leland et al., 2015). In the study on *H. americanus*, 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 the eyestalk and mesocardiac ossicle. However, this is not actually consistent with retention of the structures. If calcified material stained with calcein had been retained through the moult cycle, and the hypodermis of those structures had then secreted additional cuticle layers during growth after the stain was applied, then the calcein mark should be found more distally in the endocuticle. 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 of calcein marks in the endocuticle during the moult cycle: in five of nine specimens of *C. 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.

The most likely explanation for the detection of calcein marks in specimens which have been 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 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 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 accumulate data about chronological age.

Kilada et al. (2012) addressed the phenomenon of potentially stored and remobilized calcium carbonate as a possible cause for the detection of calcein marks in moulted specimens but argues 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 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 remobilized calcium carbonate is unlikely to be incorporated solely into the most proximal endocuticle but should be rather distributed throughout the whole layer. According to our point 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 the fate and location of a calcein mark through such a complex process (see Greenaway, 1985). Moreover, Sheridan et al. (2016) have demonstrated the persistence of calcein marks in gastric mill ossicles through the moult in *N. norvegicus* despite that the trans-moult retention of the same ossicles has been clearly disproved by the same authors. In conclusion, the trans-moult calcein detection should not be regarded as a reliable tool to provide evidence for the retention of cuticle structures before we have achieved a deeper understanding on the interaction of resorption and 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.

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.

A general relationship between cuticle thickness and body size is obvious when comparing 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 relationship is not clear. The thickness of the cuticle also varies during the different episodes of the moult cycle; most obviously, the cuticle is thinner immediately after the moult before the endocuticle deposition is completed. Unpublished data suggests the allometry of the thickness 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 *Chionectes opilio* (pers. com. Bernard Sainte-Marie, 2018). A possible explanation for this phenomenon is that the endocuticle 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 to a daily cycle. In insects, non-lamellate cuticle is laid down during the day and lamellate cuticle deposited during the night, those layers are shown as dark and light cuticle bands (Neville, 1965; Wiedenmann et al., 1986; Lukat et al., 1989; Weber, 1985; Ito et al., 2008). The images of insect endocuticle bands in these studies strongly resemble the cuticle bands in crustaceans. In some insects, these cuticle bands record number of days that passed after the last moult during cuticle hardening. Endocuticle formation can take 2-3 weeks in locusts (Neville, 1965), and once this process is completed, no further layers are added.

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

4.6 Conclusions and future directions

Since the first proposal of direct age determination for crustaceans through presumed annual growth bands in gastric mill ossicles, this method has been increasingly used (e.g. Clore, 2014; 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 (Leland et al., 2011), without considering the striking differences in growth mechanisms. In non-crustacean organisms that possess annual or otherwise periodic bands or rings, growth is necessarily a continuous process, such that seasonal conditions that influence metabolism are reflected in differentiated growth increments. Growth in crustaceans is profoundly different from 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 process, and body size increase is only possible through moulting. Studies using presumed growth 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 that might be held responsible for the formation of bands in the endocuticle.

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 anatomical ageing structures during the moult should be studied. An explanation for the close match of band counts with age estimates based on growth models (Kilada et al., 2015; 2017), might arise with a deeper understanding of the underlying processes in the formation of bands in the endocuticle. There are certainly bands that can be observed in the endocuticle, but we contend they do not represent annual growth increments that indicate chronological age. However, the 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 plywood structure of deposited chitin layers (Fabritius et al., 2016), but these are finer-scale structures than bands that have been attributed to ageing. Future studies on the mechanistic cause for this broader layering may reveal what actually forms the observed bands. The control and rhythmicity of the process of postmoult endocuticle deposition might be responsible for the 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 crustacean species are actually retained through the moult. Future research should therefore focus on understanding the process of postmoult endocuticle deposition and mineralisation in additional species of crustaceans.

Acknowledgements

This project was funded by the UK Sea Fish Industry Authority's (Seafish) Strategic Investment Programme 2015-18. Co-funding was received from Kilkeel/Whitby Seafoods (UK). We would like to personally thank Lynn Gilmore from Seafish and Daniel Whittle from Whitby Seafoods for their support. We thank Lorraine McLean (QUB) and Simon Exley for their help in obtaining live specimens, as well as fishermen Paul Magean, Frank Rogers, Brian, Mark and Alan Chambers for additional specimens. We acknowledge Lawrence Eagling and Emma Gorman (both QUB) for their help with maintaining specimens and regularly checking tanks. We also thank Kristin Mahlow (Museum für Naturkunde, Berlin, Germany) for her help with the micro-computed tomography scan and Dr Dula Parkison (Lawrence Berkeley National Laboratory) for supporting synchrotron μ -CT scanning and reconstruction.

Bibliography

- Abele, D., Brey, T. and Philipp, E., 2009. Bivalve models of aging and the determination of molluscan lifespans. *Exp. Gerontol.* 44, 307-315.
- Brösing, A., 2002. Die Magenstrukturen der Brachyura (Crustacea, Decapoda): Morphologie und phylogenetische Bedeutung. Humboldt-Universität zu Berlin.
- Brösing, A., 2008. A reconstruction of an evolutionary scenario for the Brachyura (Decapoda) in the Context of the Cretaceous-Tertiary boundary. *Crustaceana* 81, 271-287.
- Brösing, A., 2014. Foregut structures of freshly moulted exuviae from *Maja crispata*, *Cancer pagurus* and *Pseudosesarma moeschi* (Decapoda: Brachyura). *J. Nat. Hist.* 48, 543-555.
- Brösing, A., Richter, S. and Scholtz, G., 2002. The foregut-ossicle system of *Dromia wilsoni*, *Dromia personata* and *Lauridromia intermedia* (Decapoda, Brachyura, Dromiidae), studied with a new staining method. *Arthropod Struct. Dev.* 30, 329-338.
- Brösing, A., Richter, S. and Scholtz, G., 2007. Phylogenetic analysis of the Brachyura (Crustacea, Decapoda) based on characters of the foregut with establishment of a new taxon. *J. Zool. Syst. Evol. Res.* 45, 20-32.
- Brösing, A. and Türkay, M., 2011. Gastric teeth of some thoracotreme crabs and their contribution to the brachyuran phylogeny. *J. Morphol.* 272, 1109-1115.
- Campana, S.E., 2001. Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *J. Fish Biol.* 59, 197-242.
- Carmichael, S.W., 2013. Look in a microscope to determine the age of a shrimp, crab, or lobster! *Micros. Today* 21, 8.
- Castejón, D., Ribes, E., Durfort, M., Rotllant, G. and Guerao, G., 2015a. Foregut morphology and ontogeny of the mud crab *Dyspanopeus sayi* (Smith, 1869) (Decapoda, Brachyura, Panopeidae). *Arthropod Struct. Dev.* 44, 33-41.
- Castejón, D., Rotllant, G., Ribes, E., Durfort, M. and Guerao, G., 2015b. Foregut morphology and ontogeny of the spider crab *Maja brachydactyla* (Brachyura, Majoidea, Majidae). *J. Morphol.* 276, 1109-1122.
- Clore, J.L., 2014. Investigation of the definitive age of freshwater decapod crustaceans, *Orconectes propinquus* and *Procambarus clarkii*, via growth bands in the calcified regions of the eyestalk and gastric mill. Grand Valley State University.
- Creber, G.T., 1977. Tree Rings: a natural data storage system. *Biol. Rev.* 52, 349-381.
- Davie, P.J.F., Guinot, D. and Ng, P.K.L., 2015. Anatomy and functional morphology. In: Castro, P., Davie, P.J.F., Guinot, D., Schram, F.R. and von Vaupel Klein, J.C. (Eds), *Treatise on Zoology - Anatomy, Taxonomy, Biology - The Crustacea. Decapoda-Brachyura*, Vol. 9, part C-1. Koninklijke Brill, Leiden, pp. 185-243.
- Fabritius, H.-O., Ziegler, A., Friák, M., Nikolov, S., Huber, J., Seidl, B.H.M., Ruangchai, S., Alagboso,

593 F.I., Karsten, S., Lu, J., Janus, A.M., Petrov, M., Zhu, L.-F., Hemzalová, P., Hild, S., Raabe, D., and
 594 Neugebauer, J., 2016. Functional adaptation of crustacean exoskeletal elements through
 595 structural and compositional diversity: a combined experimental and theoretical study.
 596 Bioinspir. Biomim. 11, 55006.

597 Farmer, A.S.D., 1973. Age and growth in *Nephrops norvegicus* (Decapoda: Nephropidae). Mar. Biol.
 598 23, 315–325.

599 Greenaway, P., 1985. Calcium balance and moulting in the Crustacea. Biol Rev 60, 425–454.

600 Hartnoll, R.G., 2001. Growth in Crustacea - Twenty years on. Hydrobiologia 449, 111–122.

601 Hasyima Ismail, N., Amin Safwan, A., Fairuz Fozi, N., Megat, F.H., Muhd Farouk, H., Kamaruddin,
 602 S.A., Ikhwanuddin, M., and Ambak, M.A., 2017. Study on carapace width growth band counts
 603 relationship of orange mud crab, *Scylla olivacea* (Herbst, 1796) from Terengganu coastal
 604 waters, Malaysia. Pakistan J. Biol. Sci. 20, 140-146.

605 Ibrahim, N.K. and Kilada, R., 2015. Preliminary investigation of direct age determination using
 606 band counts in the gastric mill of the blue swimmer crab (*Portunus pelagicus*) in two salt-
 607 water lakes in the Eastern Mediterranean. J. Crust. Biol. 36, 119-128.

608 Ito, C., Goto, S.G., Shiga, S., Tomioka, K., and Numata, H., 2008. Peripheral circadian clock for the
 609 cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci U.S.A. 105, 8446–
 610 8451.

611 Kilada, R. and Acuña, E., 2015. Direct age determination by growth band counts of three
 612 commercially important crustacean species in Chile. Fish. Res. 170, 134–143.

613 Kilada, R., Agnalt, A.-L., Arboe, N.H., Bjarnason, S., Burmeister, A., Farestveit, E., Gíslason, Ó.S.,
 614 Guðlaugsdóttir, A., Guðmundsdóttir, D., Jónasson, J.P., Jónsdóttir, I.G., Kvalsund, M., Sheridan,
 615 M., Søvik, G., and Stansbury, D., 2015. Feasibility of using growth band counts in age
 616 determination of four crustacean species in the Northern Atlantic. J. Crust. Biol. 35, 499–503.

617 Kilada, R. and Driscoll, J.G., 2017. Age determination in crustaceans: a review. Hydrobiologia 799,
 618 1–16.

619 Kilada, R., Reiss, C.S., Kawaguchi, S., King, R.A., Matsuda, T., and Ichii, T., 2017a. Validation of band
 620 counts in eyestalks for the determination of age of Antarctic krill, *Euphausia superba*. PLoS
 621 One 12, 1–14.

622 Kilada, R., Sainte-Marie, B., Rochette, R.R., Davis, N., Vanier, C., and Campana, S., 2012. Direct
 623 determination of age in shrimps, crabs, and lobsters. Can. J. Fish. Aquat. Sci. 69, 1728–1733.

624 Kilada, R., Webb, J.B., McNeel, K.W., Slater, L.M., Smith, Q., and Ferguson, J., 2017b. Preliminary
 625 assessment of a direct agedetermination method for 3 commercially important crustaceans
 626 from Alaska. Fish. Bull. 115, 42–49.

627 Krafft, B.A., Kvalsund, M., Søvik, G., Farestveit, E., and Agnalt, A.-L., 2016. Detection of growth zones
 628 in the eyestalk of the Antarctic krill *Euphausia superba* (Dana, 1852) (Euphausiacea). J. Crust.

629 Biol. 36, 267–273.
 630 Leland, J., and Bucher, D., 2017. Direct age determination with validation for commercially
 631 important Australian lobster and crab species: western, eastern, southern and ornate rock
 632 lobsters, and crystal, giant and mud crabs. Southern Cross University, New South Wales,
 633 Australia, September, CC by 3.0.
 634 Leland, J.C., Bucher, D.J., and Coughran, J., 2015. Direct age determination of a subtropical
 635 freshwater crayfish (redclaw, *Cherax quadricarinatus*) using ossicular growth marks. PLoS
 636 One 10, 1–11.
 637 Leland, J.C., Coughran, J., and Bucher, D.J., 2011. A preliminary investigation into the potential
 638 value of gastric mills for ageing crustaceans. In: Asakura, A. (Ed.), New Frontiers in
 639 Crustacean Biology, Proceedings of the TCS Summer Meeting, Tokyo, Japan, 20–24
 640 September 2009. Koninklijke Brill, Leiden, pp. 57–68.
 641 Lukat, R., Weber, F., and Wiedenmann, G., 1989. Cyclic layer deposition in the cockroach
 642 (*Blaberus craniifer*) endocuticle: A decentral circadian “clock”? J Insect Physiol 35, 321–329.
 643 Matthews, T., Derby, C., Maxwell, K., and Bertelsen, R., 2015. Age determination method using
 644 neurolipofuscin: a reply to Buesa. New Zeal J Mar. Freshw. Res. 49, 497–502.
 645 Maxwell, K.E., Matthews, T., Sheeny, M.R.J., Bertelsen, R., and Derby, C.D., 2007. Neurolipofuscin is
 646 a measure of age in the caribbean spiny lobster, *Panulirus argus*, in Florida. Biol. Bull. 213,
 647 55–66.
 648 Maynard, D.M., and Dando, M.R., 1974. The structure of the stomatogastric neuromuscular system
 649 in *Callinectes sapidus*, *Homarus americanus* and *Panulirus argus* (Decapoda Crustacea). Phil.
 650 Trans. R. Soc. Lond. B, Biol. Sci. 268, 161–220.
 651 Neville, A.C., 1965. Circadian organization of chitin in some insect skeletons. Q. J. Microsc. Sci.
 652 106, 315–325.
 653 O'Donovan, V., and Tully, O., 1996. Lipofuscin (age pigment) as an index of crustacean age:
 654 Correlation with age, temperature and body size in cultured juvenile *Homarus gammarus* L.
 655 J. Exp. Mar. Biol. Ecol. 207, 1–14.
 656 Reimann, A., Richter, S., and Scholtz, G., 2011. Phylogeny of the Anomala (Crustacea, Decapoda,
 657 Reptantia) based on the ossicles of the foregut. Zool. Anz. 250, 316–342.
 658 Rodhouse, P.G., and Hatfield, E.M.C., 1990. Age determination in squid using statolith growth
 659 increments. Fish. Res. 8, 323–334.
 660 Roer, R., and Dillaman, R., 1984. The structure and calcification of the crustacean cuticle. Am. Zool.
 661 24, 893–909.
 662 Sheridan, M., O'Connor, I., and Henderson, A.C., 2016. Investigating the effect of molting on gastric
 663 mill structure in Norway lobster (*Nephrops norvegicus*) and its potential as a direct ageing
 664 tool. J. Exp. Mar. Biol. Ecol. 484, 16–22.

- Sheridan, M., and O'Connor, I., 2018. Evidence of complete gastric mill ossicle loss at ecdysis in the European green crab *Carcinus maenas* (Linnaeus, 1758) (Decapoda: Brachyura: Carcinidae). J. Crust. Biol. (in print) <https://doi.org/10.1093/jcbiol/ruy045>.
- Sheridan, M., Officer, R., O'Connor, I., and Lordan, C., 2015. Investigating the feasibility of using growth increments for age determination of Norway lobster (*Nephrops norvegicus*) and brown crab (*Cancer pagurus*). J. Crust. Biol. 35, 495–498.
- Tang, F., Minch, T., Dinning, K., Martyniuk, C.J., Kilada, R., and Rochette, R., 2015. Size-at-age and body condition of juvenile American lobsters (*Homarus americanus*) living on cobble and mud in a mixed-bottom embayment in the Bay of Fundy. Mar. Biol. 162, 69–79.
- Vatcher, H.E., Roer, R.D., and Dillaman, R.M., 2015. Structure, molting, and mineralization of the dorsal ossicle complex in the gastric mill of the blue crab, *Callinectes sapidus*. J. Morphol. 276, 1358–1367.
- Vogt, G., 2012. Ageing and longevity in the Decapoda (Crustacea): A review. Zool Anz 251, 1–25.
- Wahle, R.A., and Fogarty, M.J., 2006. Growth and development: Understanding and modelling growth variability in lobsters. In: Phillips, B.F. (Ed.), Lobsters: Biology, Management, Aquaculture and Fisheries. Blackwell Publishing Ltd, Oxford, pp. 1–44.
- Weber, F., 1985. Postmolt cuticle growth in a cockroach: in vitro deposition of multilamellate and circadian-like layered endocuticle. Experientia 41, 398–400.
- Welinder, B.S., 1974. The crustacean cuticle—I. Studies on the composition of the cuticle. Comp. Biochem. Physiol., Part A Physiol. 47, 779–787.
- Welinder, B.S., 1975a. The crustacean cuticle-II. Deposition of organic and inorganic material in the cuticle of *Astacus fluviatilis* in the period after moulting. Comp. Biochem. Physiol., Part B Biochem. 51, 409–416.
- Welinder, B.S., 1975b. The crustacean cuticle-III. Composition of the individual layers in cancer pagurus cuticle. Comp. Biochem. Physiol., Part A Physiol. 52, 659–663.
- Wiedenmann, G., Lukat, R., and Weber, F., 1986. Cyclic layer deposition in the cockroach endocuticle: A circadian rhythm? J. Insect Physiol. 32, 1019–1027.

Supporting information

Ethics approval

The conducted research adhered to legal requirements for animal care in the European Union.

Consent for publication

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**

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

704 **Declaration of interest**

705 none

706 **Funding**

707 This project was funded by the UK Sea Fish Industry Authority's (Seafish) Strategic Investment
708 Programme 2015-18. Co-funding was received from Kilkeel/Whitby Seafoods (UK).

709 **Author Contributions**

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

715

716 **Figure captions**

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

722 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
724 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 cuticle lamellae which do not correspond to the cuticle bands.

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 with carapace removed to expose the gastric mill and other organs (carapace length 115 mm). (b) Dorsal and (c) ventral aspect of intermoult gastric mill after ventro-longitudinal incision to expose ossicles. The unpaired urocardiac ossicle lies centrally and carries the median tooth plate. Adjoining anteriorly is the transversely elongated mesocardiac ossicle, flanked on each side by the paired pterocardiac ossicles which are connected to the zygo-cardiac ossicles that carry the lateral tooth plates. (d) The mesocardiac, zygo-cardiac and pterocardiac ossicles were found loose in the stomach content of freshly moulted specimens. Two smaller, unassigned ossicles were also present. The black asterisks in (a), (b) and (c) label the same structure to facilitate orientation; Abbreviations: gm – gastric mill, he – heart, lt - lateral tooth plate, mc - mesocardiac ossicle, mt - median tooth plate, ov – ovary, pc - pterocardiac ossicle, uc - urocardiac ossicle, zc - zygo-cardiac ossicle.

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

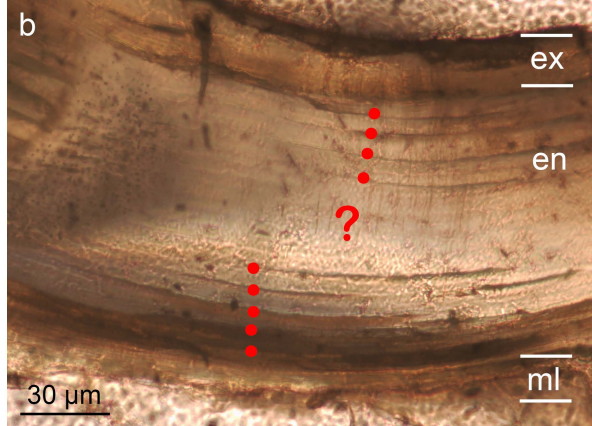
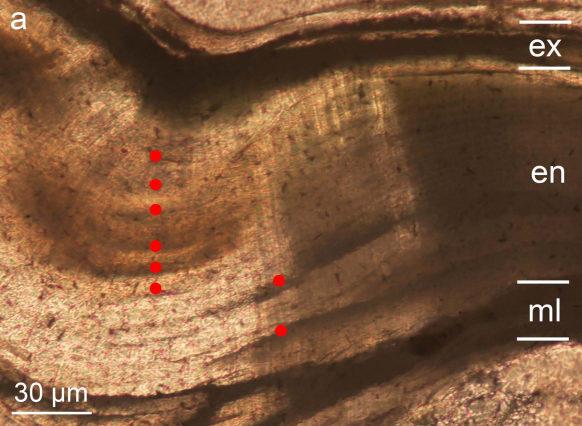
Fig. 5 Micro-computed tomography of anterior thorax of freshly moulted Norway Lobster *Nephrops norvegicus*. Volume rendering viewed with AMIRA software (“volren glow” colour scheme), longitudinally cut using orthoslice tool. The lateral tooth plate, mandible and the eye of the left body half are shown. 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.

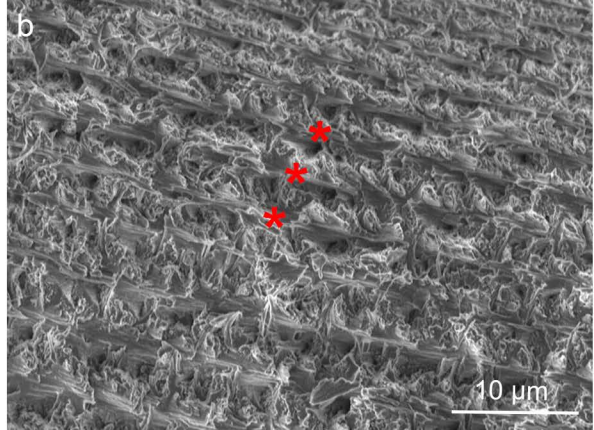
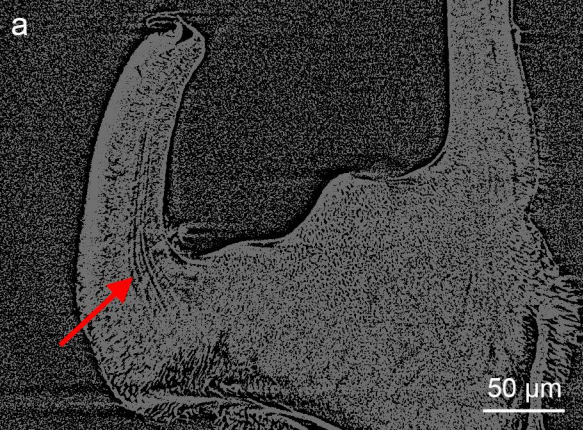
Fig. 6 The gastric mill during the moult in Velvet Crab *Necora puber*. (a) Exuvia of male with dorsal carapace folded open along the moult line (carapace width 45 mm). (b) Ventral view on dissected gastric mill in exuvia showing that teeth are moulted but calcified parts of ossicles are missing. (c)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

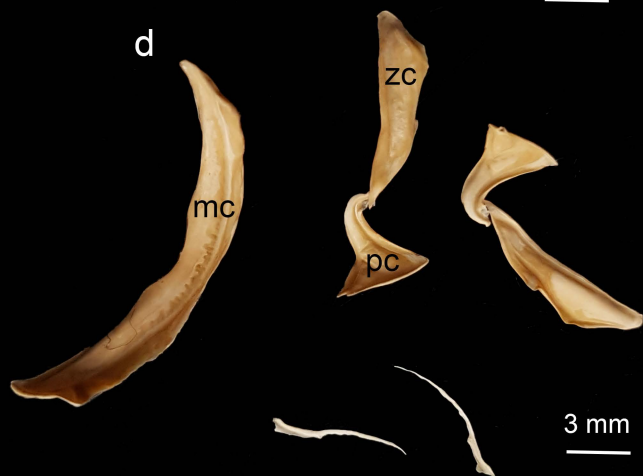
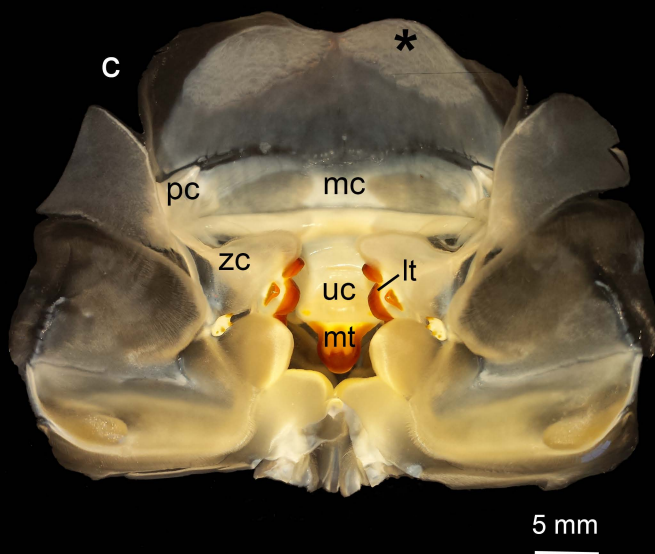
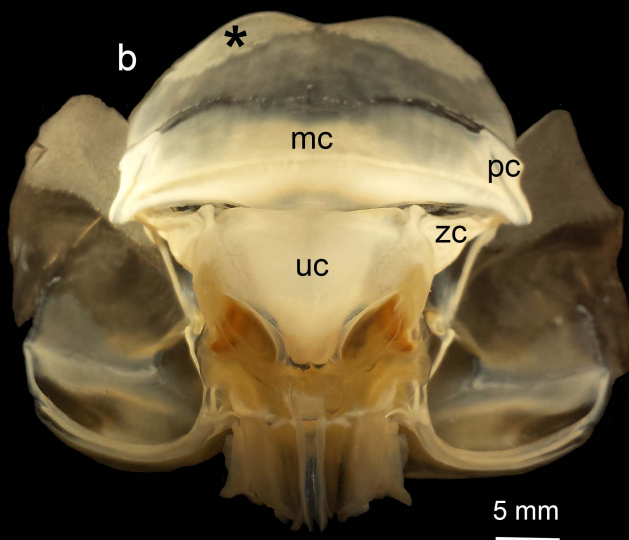
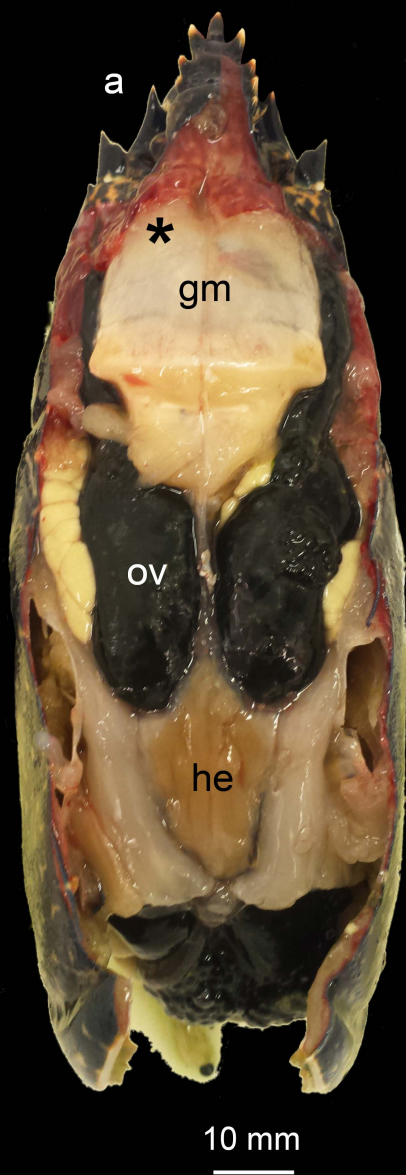
translucent ossicles (same aspect). (e) Ossicles found in stomach of freshly moulted specimen. Abbreviations: lt - lateral tooth plate, mc - mesocardiac ossicle, mt - median tooth plate, pc - pterocardiac ossicle, uc - urocardiac ossicle, zc - zygo-cardiac ossicle.

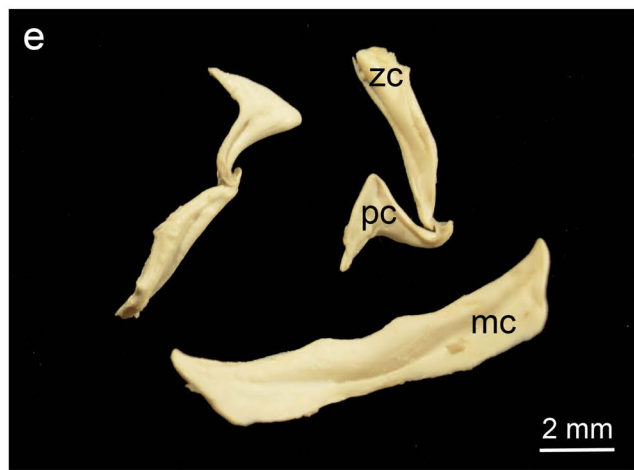
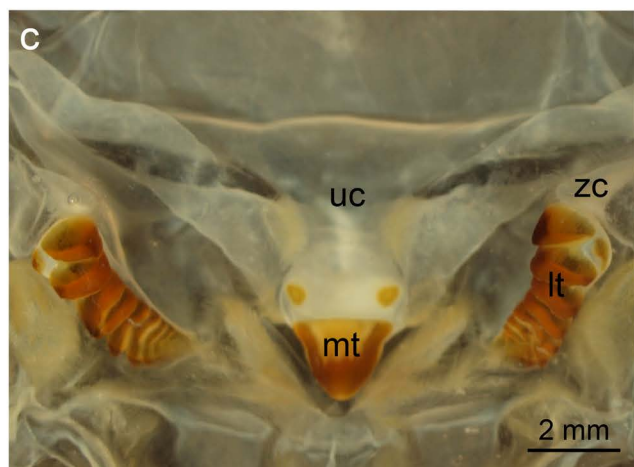
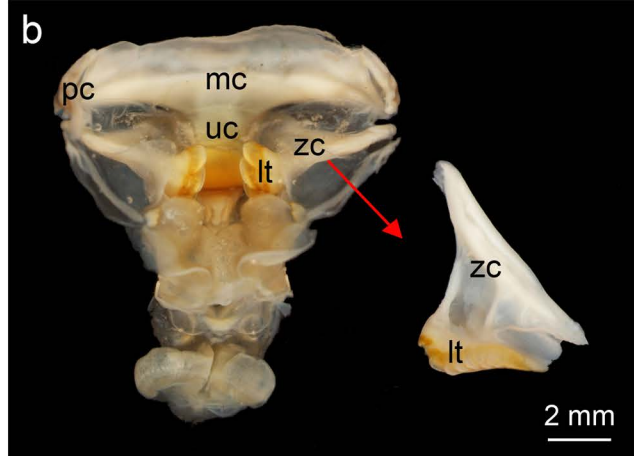
Fig. 7 The gastric mill during the moult in Brown Crab *Cancer pagurus*. (a) Exuvia (top, carapace width 124 mm) of and emerged specimen (bottom, carapace width 149 mm) of male. (b) Ventral view on dissected gastric mill showing calcified ossicles. (c) Gastric mill of freshly moulted specimen showing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

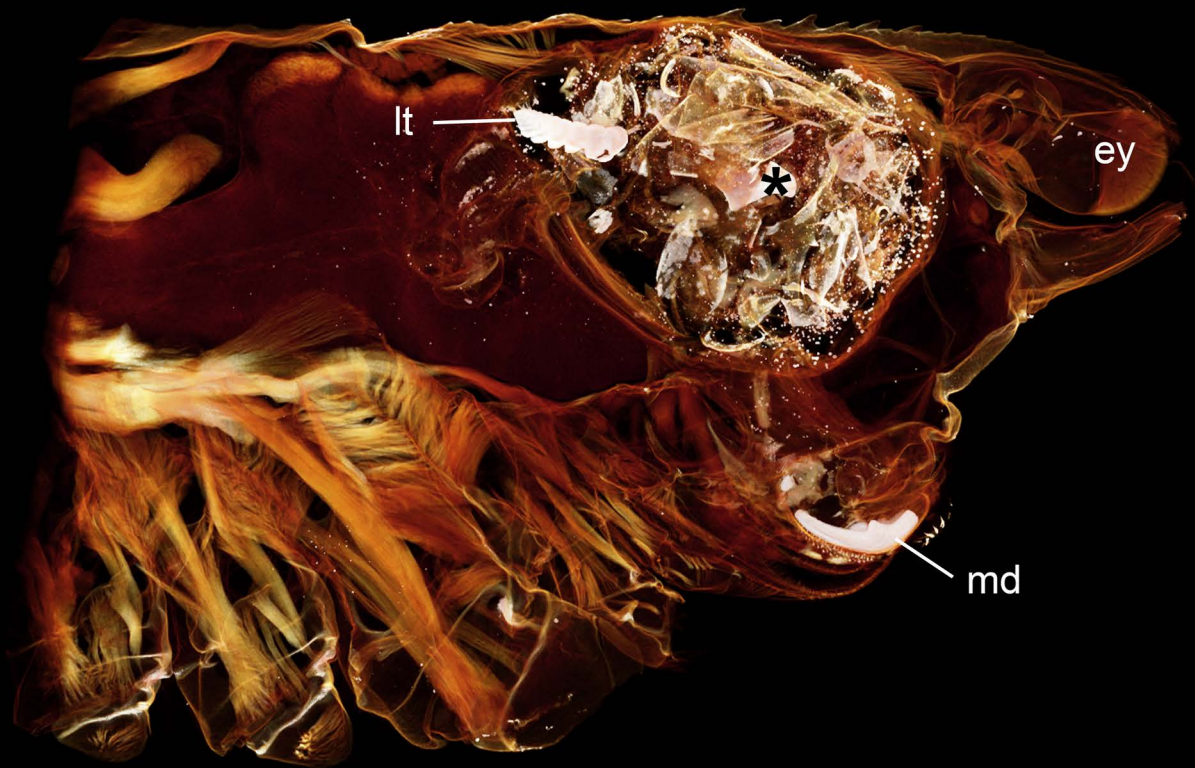
Fig. 8 The zygo-cardiac ossicle in the Norway Lobster *Nephrops norvegicus* shortly before moulting in histological sections. (a) Close to the moult, the old cuticle of the gastric tooth in the zygo-cardiac ossicle (white label zc) is already delaminated from the newly formed tooth (black label zc). (b) The shed cuticle consists of all three cuticle layers: endo-, exo- and epicuticle. (c) The newly 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 turquoise, which overlies the cuticle epithelium. The endocuticle is not formed until after the moult. (e) The basal part of the zygo-cardiac ossicle showing the old cuticle (white label zc) detached from the newly formed cuticle and its underlying hypodermis (black label zc). (f) The 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 thin red-staining epicuticle, a turquoise and red staining exocuticle and a thick light turquoise staining endocuticle. The endocuticle contains bands (indicated by white asterisks) and will be completely shed at the upcoming moult. Abbreviations: en – endocuticle, ep – epicuticle, ex – exocuticle, gt – gastric teeth, hd – hypodermis, zc – zygo-cardiac ossicle.

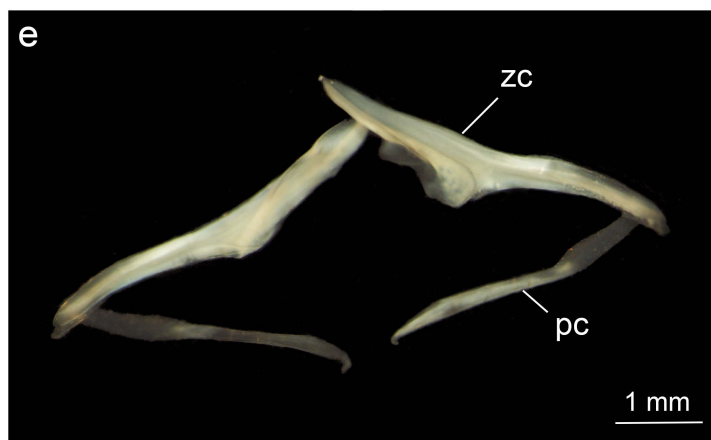
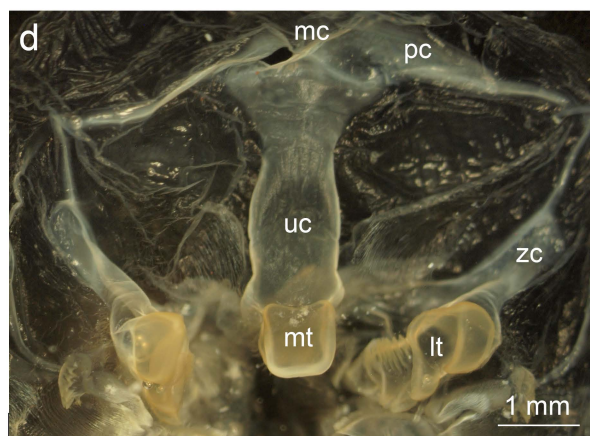
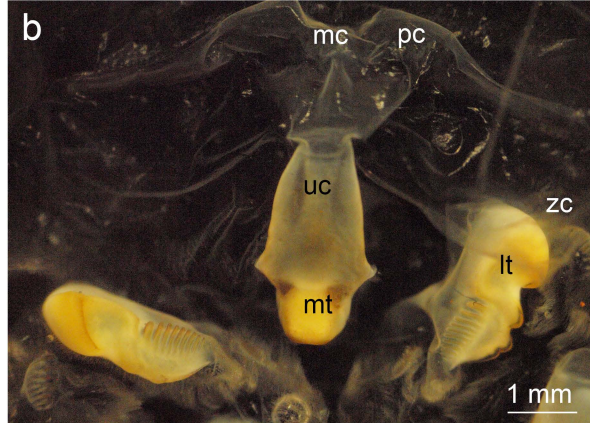






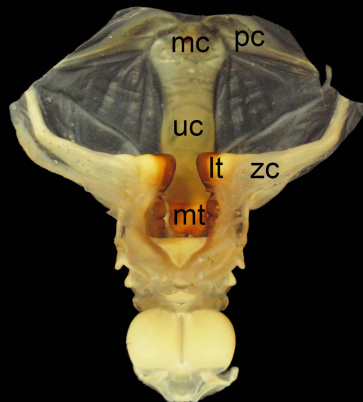




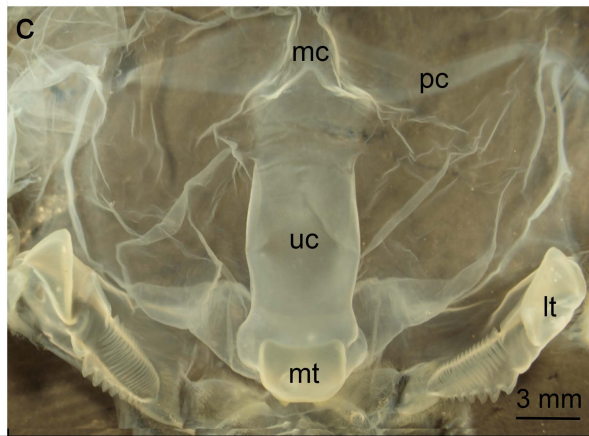


a

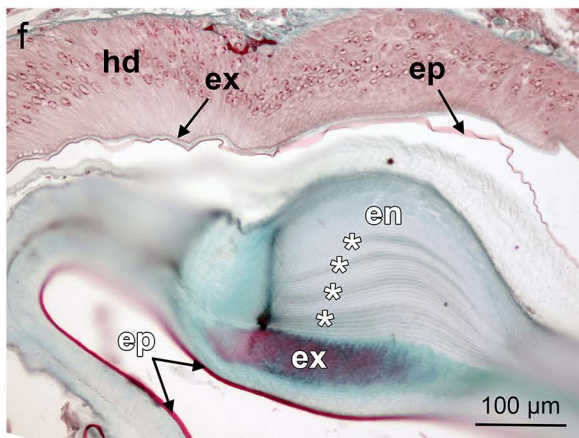
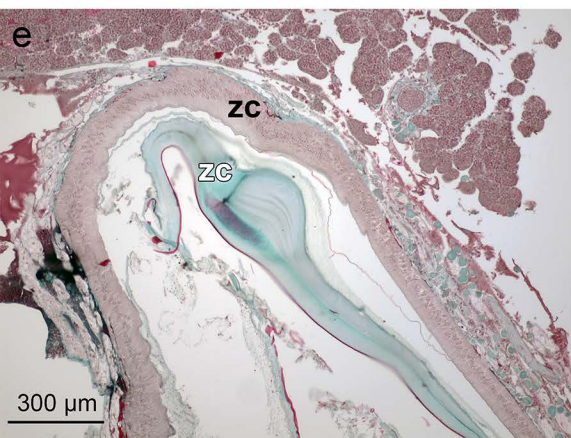
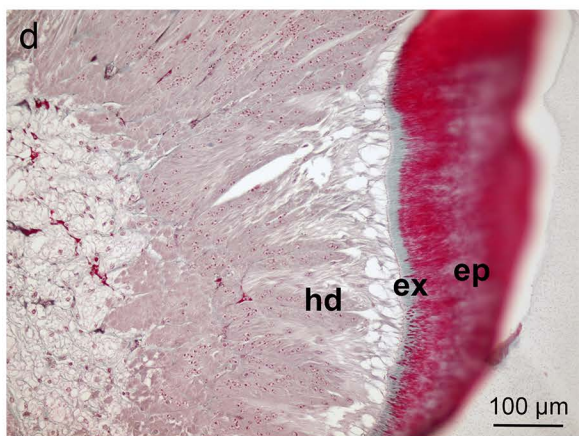
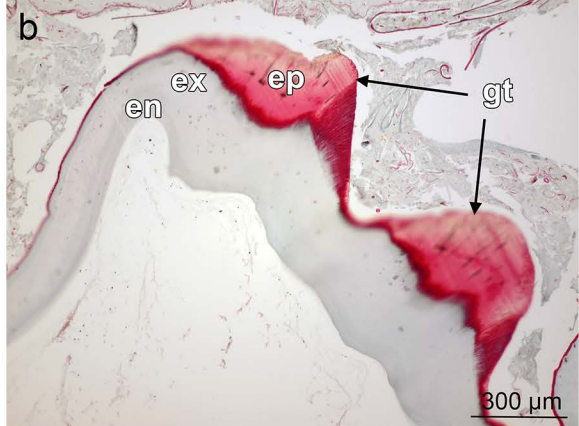
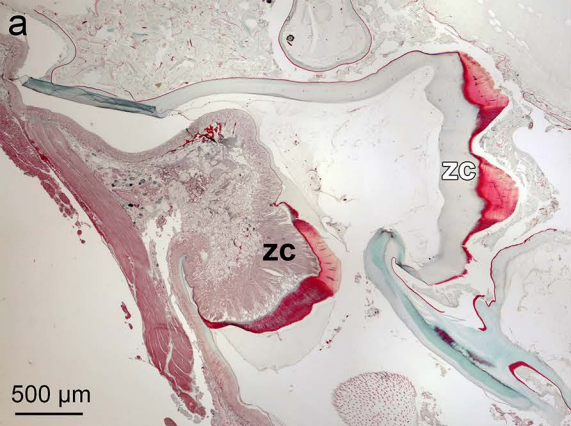
20 mm

b

10 mm

c

3 mm



Freshly moulted Norway Lobster (*Nephrops norvegicus*)

