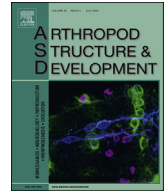




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The crustacean cuticle does not record chronological age: New evidence from the gastric mill ossicles

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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 chronological age, meaning it is unreliable for determining crustacean age.

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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 and 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.

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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, 2015, 2017b; Kilada and Acuña, 2015). 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).

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., 2015). 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 zygo-cardiac and uro-cardiac 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 heli-coidal 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 and 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), *N. 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

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