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**Detection of growth zones in the eyestalk of the Antarctic krill
Euphausia superba (Dana, 1852) (Euphausiacea)**

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DETECTION OF GROWTH ZONES IN THE EYESTALK OF THE ANTARCTIC KRILL *EUPHAUSIA SUPERBA* (DANA, 1852) (EUPHAUSIACEA)

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ABSTRACT

No reliable measures of age currently exist in the Antarctic krill, *Euphausia superba* (Dana, 1852). The eyestalks from 51 individuals were dissected, cut in longitudinal sections and studied for identifying growth zones. The krill was collected at the South Orkney Islands during January and February 2015, and varied between 30 and 53 mm in total body length. Up to six growth zones were identified, each zone consisting of one light and one dark section. The width of the longitudinal sections increased with increasing body length, although there were differences between sexes. Females tended to have narrower growth zones from the third zone and onwards compared with males. Data show that male subadult stages (MIIA1, MIIA2 and MIIA3) had 2.2 ± 0.8 (average \pm SD) zones and adult male stages had 3.8 ± 0.8 zones. The female juvenile stage (FIIB) had 1.7 ± 0.5 zones and adult females (FIIIA-E) had 3.7 ± 1.0 zones. There were positive relationships between the number of zones and the maturity stage, and between the number of zones and body length. Further knowledge about molting process in the Antarctic krill and a verification of the ageing procedure from krill with a known age is needed before the number of growth zones can be definitely established as an indicator of age. The detection of growth zones in the Antarctic krill will be an important contribution to the understanding of the biology of the species if the zones actually represent annual growth.

KEY WORDS: age determination, Antarctica, fishery management, Southern Ocean

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INTRODUCTION

Knowledge about age and age distribution of a fishery targeted species are standard indicators used to assess the status of the stock in the development of sustainable management practices (Iversen, 1996). The Antarctic krill *Euphausia superba* Dana, 1852, with its vast cold water circumpolar habitat (Atkinson et al., 2006; Tarling et al., 2006), is an abundant fishery resource; however, no reliable estimates of age in this species currently exist (Nicol, 2000; Nicol et al., 2012).

Length-frequency methods have been used to determine age groups of krill (Ruud, 1932; Marr, 1962; Ivanov, 1970; Mackintosh, 1973), but different interpretations of the data give varying results. Like many other euphausiids, the Antarctic krill is capable of shrinking when starved (Ikeda and Dixon, 1982; Quetin and Ross, 1991; Auerswald et al., 2015). This strategy reduces metabolic energy costs associated with a larger body, and could be beneficial when residing in habitats and/or seasons marked by food scarcity. Such a strategy is still not accepted for all krill stocks because food availability does vary within its vast distributional range (Nicol, 2000). Assuming that shrinking through molting is the overall norm, however, the conclusions reached by morphological ageing could be questionable. The use of alternative methods include biochemical assays of the fluorescent age pigment (FAB) (Ettershank, 1983) in combination with length measurements, but this method is regarded as not practical due to the labor-intensive pigment extractions (see Nicol, 1987). The crystalline cone number of the com-

pound eye has also been examined as a possible indicator of age (Sun et al., 1995). This method depends on the eye diameter being unaffected by shrinkage, but a reduction in eye diameter has been shown to occur (Shin and Nicol, 2002).

The method of using histological quantification of lipofuscin has been applied on several species of shrimps, crabs, and lobsters (Sheehy et al., 1998; Uglem et al., 2005; Kodama et al., 2006; Maxwell et al., 2007). Because lipofuscin accumulation is influenced by environmental factors such as temperature, this method is likely a better indicator of physiological age rather than actual age (Wahle et al., 1996; Vogt, 2012). There are also some limitations with this method in connection with the quality of the readability of these structures, as they seem highly sensitive to fixation treatments (Nicol, 1987).

It is still unclear whether some crustacean hard structures, which potentially could contain age information, are retained across molts. Based on histological examinations of the mesocardiac ossicle of the gastric mill of the blue crab (*Callinectes sapidus* Rathbun, 1896), Vatcher et al. (2015) concluded that all calcified structures are shed completely. Brösing (2014), however, demonstrated interspecific differences in the presence of ossicles in the exuviae. Furthermore, he described varying degrees of calcification in some structures (even in the urocardiac and mesocardiac regions), indicating that some parts could remain through molts. A recent technique has recognized features assumed to be growth increments, bands or zones in the cuticle of a number of species, e.g., Norway lobster (*Nephrops norvegicus* Lin-

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naeus, 1758), European and American lobsters (*Homarus gammarus* Linnaeus, 1758 and *H. americanus* H. Milne Edwards, 1837), a deep-water shrimp (*Pandalus borealis* Krøyer, 1838) and the blue swimming crab (*Portunus pelagicus* Linnaeus, 1758) (Leland et al., 2011, 2015; Kilada et al., 2012, 2015; Kilada and Ibrahim, 2016). Calcein-staining experiments performed by Kilada et al. (2012) indicated that the cuticle of the eyestalk appears to be shed but the stain is still retained in the postmolt eyestalk. Leland et al. (2015) also demonstrated the retention of calcein tags in ossicles. No clear explanations of how these structures are retained across molts has been put forward.

The purpose of this study was to modify the ageing technique described by Kilada et al. (2012) and Leland et al. (2015) to assess its applicability on the small eyestalk of the Antarctic krill for the identification of potential growth zones applicable as an age indicator for this species. This technique has the potential of being implemented as a standard for direct age determination for this species if such growth zones were found and future investigations demonstrate retention through molts of these growth zones in the eyestalk.

MATERIALS AND METHODS

Antarctic krill was collected at the South Orkney Islands (60°35'S, 45°30'W) during January to February 2015 in conjunction with a synoptic survey run annually by the Institute of Marine Research, Norway. Collections were made using a 7 mm mesh (stretched) survey trawl ("Macroplank-

ton trawl"; Krafft et al., 2010) hauled from FV *Juvel* (Olympic AS), a commercial Norwegian ramp trawler, from a depth of 200 m to the surface at stations evenly distributed over the study area. Individual Antarctic krill were sampled from the catch to represent the various developmental stages present. Samples were preserved on 70% ethanol and detailed measurements were made of sex and maturity stages using the classification methods outlined by Makarov and Denys (1981) and described in Krafft et al. (2015). Males were separated into three sub adult stages: MIIA1 (petasma vesicles are not divided, but appear as a small "bump" or "bubble" at the root), MIIA2 (petasma has developed the "bubble" to a split with one or two "fingers"), and MIIA3 (petasma root with two short "fingers" and an incipient formation of "wings" on the opposite hold), and two adult stages: MIIIA (petasma fully developed, with swollen "fingers" and with a "wing" overlap, ductus ejaculatori are also visible ventrally, but these are sealed and spermatophores cannot be squeezed out), and MIIIB (petasma as for MIIIA, ductus ejaculatori has spermatophores that can be pressed out, or with the duct passage open where spermatophores are already deposited). Females were separated into one sub adult stage: FIIB (thelycum is small and colorless), and five adult stages: FIIIA (thelycum is fully developed for spawning, red-pigmented and strongly chitinated), FIIIB (thelycum as FIIIA but fertilized with spermatophores), FIIIC (also with spermatophores, mature eggs or large ovaries visible under carapace, but carapace is not swollen), FIIID (with spermatophores, carapace is swollen and this swelling extends into the first abdominal segment), and FIIIE (fully spawned, the ovaries are small and the carapace is hollow). Juveniles, unlike all other stages, have no visible sexual characteristics (no visible petasma or thelycum).

Total body length was measured (± 1 mm) from the anterior margin of the eye to the tip of the telson excluding the setae, according to the "Discovery method" (Marr, 1962). The diameter of each eye was measured using a caliper. Eyestalks were dissected under a dissecting scope and fixed in a 4% glycerol/26% water/70% ethanol solution for more than 24 hours and the soft tissue was then removed by gentle rinsing. The cleaned eyestalks (Fig. 1A) were molded into an epoxy resin (Aeropoxy

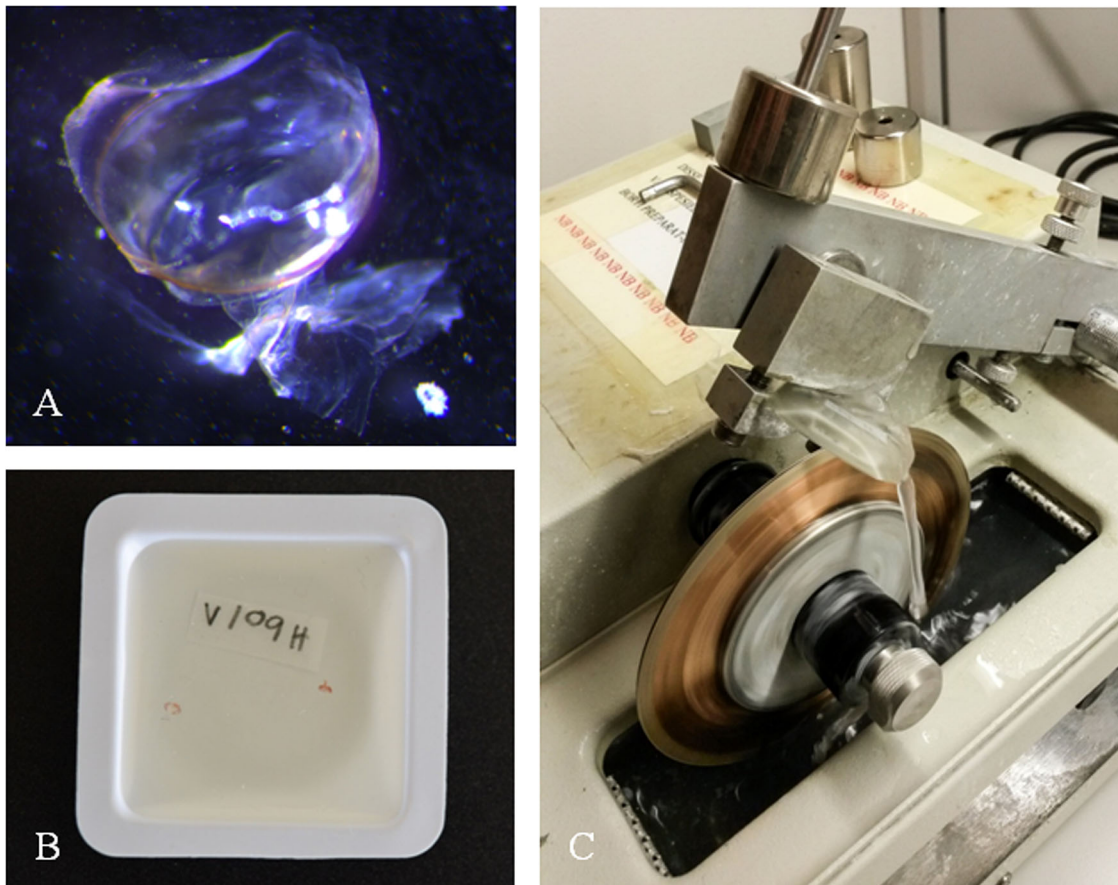


Fig. 1. (A) Cleaned eyestalk from the Antarctic krill *Euphausia superba*. Left and right eyestalks molded into an epoxy resin (B) and longitudinal sectioning of each eyestalk using a double diamond-bladed ISOMET saw (C).

101 Harpiks), and left to dry for more than 72 hours at room temperature (Fig. 1B). After drying, multiple longitudinal sections (2 to 3) from each eyestalk were made using a double diamond-bladed, low speed saw (ISOMET, with low carbon blades series 15LL diameter 102 mm \times 0.3 mm and a 41 μ m spacer) (Fig. 1C). The sections were dipped in ethanol to improve the readability of structures. Despite the rigid setup with the double diamond blades for cutting through the epoxy casts, we obtained sections with a range of 50–380 μ m in thickness. The sections found most optimal for reading ranged in thickness from 100 to 228 μ m, with a mean \pm SD thickness of 165 \pm 31 μ m. A Zeiss Axioscope 2 plus using 4 \times , 40 \times and 63 \times magnifications was used to view the sections. The 4 \times magnification was found useful for an overview of the entire section, and the 40 \times and 63 \times magnifications were used for the actual counting of the growth zones (Fig. 2A, B). We found it necessary to visually search along the entire sections under the microscope to identify the parts with the cleanest cuts best suited for reading. Photos were taken with a Nikon digital sight DS-5M-U1 camera attached to the microscope. Images were captured using the NIS elements F 2.3 software and enhanced using Adobe Photoshop 12.0.4x32 to increase the contrast between adjacent growth zones.

Each eyestalk section was read by two to four readers, of which two were experienced fish-otolith readers. Growth zones were identified as paired light and dark zones (equivalent to opaque and hyaline zones in fish otoliths) (Fig. 2). The zones were counted from the basal to the distal region of the section. The width of the eyestalk section (basal to distal region) was measured, in mm, by one of the readers, as was the distance from the membranous layer to each growth zone. Calibrated measurements were made in Adobe Photoshop.

Analysis of variance (ANOVA, General Linear Model (GLM)) was applied to test statistical differences between readers, eye diameter, width of zones, number of zones in right vs. left eye, and zones vs. sex and developmental stages. Morphological measurements were included as predictor variables and the number of zones observed in the eyestalk as the response variable in a Generalized Additive Model (GAM) nonparametric regression analysis, using the Gaussian distributional fit (SAS Institute, Cary, NC, USA).

RESULTS

We prepared the eyestalks from 52 individuals and were able to count growth zones in 51. All eyestalk sections were examined by a minimum two and a maximum four readers. A maximum of six growth zones were identified by one reader in one of the males, whereas two readers could only identify five zones. There was no significant difference between the readers' counts (GLM $F = 0.43$, $p = 0.73$). In the entire sample, the mean diameter of the right eye was 0.8 \pm 0.1 mm, whereas the left was 0.9 \pm 0.7 mm, a difference that was not significant (GLM t -value = 0.96, $p = 0.34$) (Table 1). Neither did we find any difference between the right and left eyes in individual krill in term

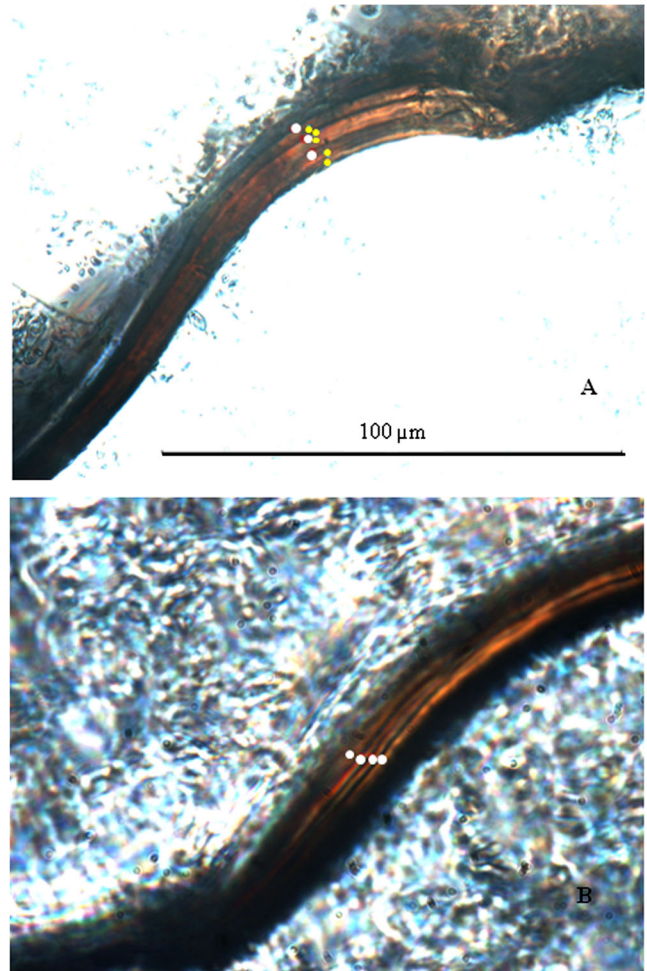


Fig. 2. Examples of potential annual growth zones of *Euphausia superba* recognized on different endocuticles as light (opaque) longitudinal fields paired by darker and thinner fields (hyaline), here represented with three zones from a subadult male (34 mm total length) at stage MIIA1 (A) and four zones from an adult female at stage FIIID (48 mm total length) (B) (highlighted with white circles). The yellow circles in A represents the location of thinner "secondary" zones. Both images are captured using 63 \times magnification; same scale in A and B.

Table 1. Sample size of the Antarctic krill *Euphausia superba* with their respective sexual maturity stages, number of eyestalk growth zones, total length, and eye diameter.

Stage	N	No. of growth zones (mean \pm SD)	Total length (mm) (mean \pm SD)	Eye diameter (mm) (mean \pm SD)
Male subadult MIIA1	5	2.0 \pm 0.7	32.4 \pm 1.8	0.7 \pm 0.0
Male subadult MIIA2	8	2.1 \pm 0.8	34.6 \pm 2.6	0.8 \pm 0.1
Male subadult MIIA3	3	2.7 \pm 0.9	37.7 \pm 1.2	0.9 \pm 0.1
Male adult MIIIA	7	3.6 \pm 0.6	47.4 \pm 2.8	0.9 \pm 0.1
Male adult MIIIB	5	4.0 \pm 1.0	48.4 \pm 3.0	1.0 \pm 0.1
Female subadult FIIB	5	1.7 \pm 0.5	34.0 \pm 0.7	0.7 \pm 0.1
Female adult FIIIA	6	3.1 \pm 0.7	39.7 \pm 2.5	0.8 \pm 0.1
Female adult FIIIB	4	3.5 \pm 0.8	45.5 \pm 5.5	0.9 \pm 0.1
Female adult FIIIC	1	3.0 \pm 0.0	46.0	1.0 \pm 0.1
Female adult FIIID	3	3.5 \pm 0.5	46.0 \pm 2.0	0.9 \pm 0.0
Female adult FIIIE	4	4.7 \pm 1.1	48.8 \pm 3.6	1.0 \pm 0.1

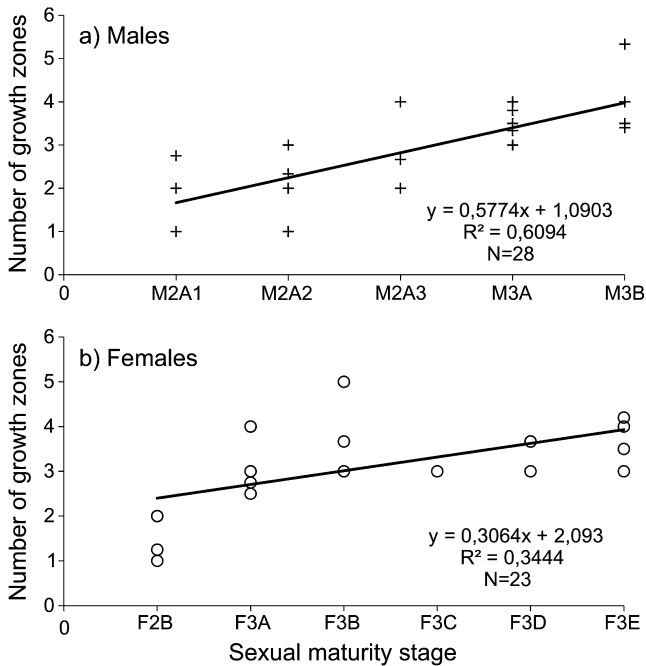


Fig. 3. Relationships between average number of zones counted in the eyestalk of the Antarctic krill *Euphausia superba* against their maturity stages in (a) males and (b) females (several readers).

of the number of growth zones counted (GLM $F = 0.02$, $p = 0.89$). There was nevertheless a significant difference in the number of growth zones between the subadult stages and the adults (GLM $F = 61.36$, $p < 0.0001$) (Fig. 3, Table 1), although with a weaker correlation for the females (Fig. 3). The male subadult stages (M2A1, M2A2 and M2A3) had on average 2.2 ± 0.8 (SD) growth zones whereas the adult male stages had 3.8 ± 0.8 growth zones. The female juvenile stage (F2B) had 1.7 ± 0.5 zones and the adult female stages (F3A-E) had 3.7 ± 1.0 growth zones (Table 1). No correlation was found between eye diameter and the number of growth zones (t -value = -0.41 , $p = 0.68$). Total length was significantly correlated with the number of growth zones (GAM t -value = 4.16 , $p = 0.0001$) (Fig. 4). No juvenile stages were identified in the sample.

The total width of the longitudinal section of the eyestalk increased with increasing total body length (GAM t -value = 7.83 , $p < 0.0001$). There was a tendency for females to have narrower growth zones than males (Fig. 5). The widths

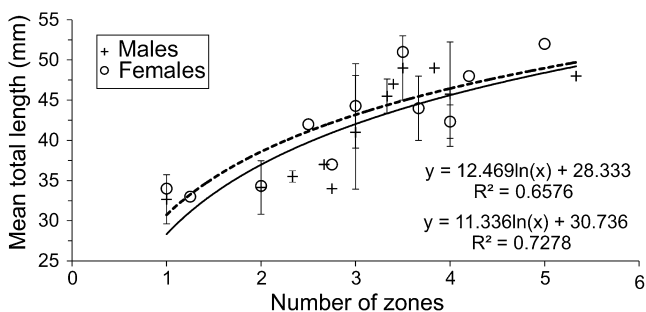


Fig. 4. The relationship between total body length against average number of zones counted (measurements made by several readers) in the Antarctic krill *Euphausia superba*.

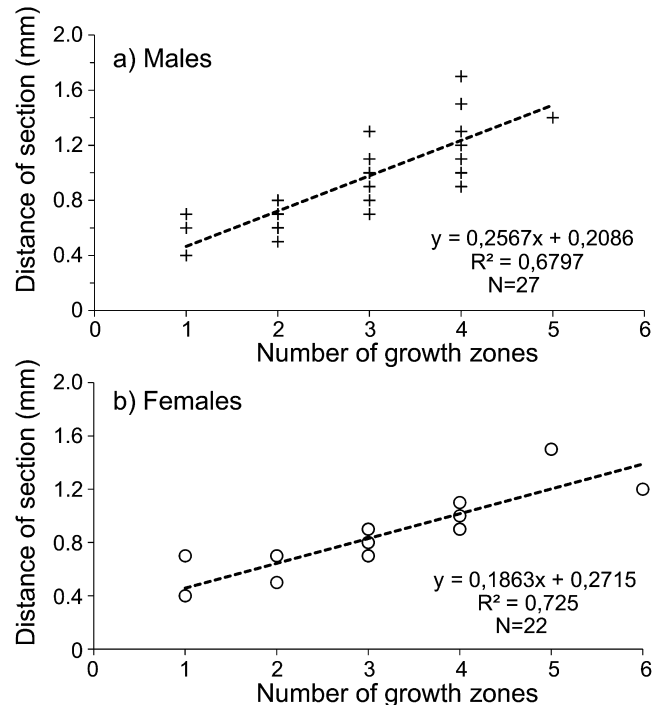


Fig. 5. Total distance of the width of the eyestalk longitudinal section (mm) and number of growth zones found in the Antarctic krill (a) males and (b) females (measurements made by one reader).

of the first and second zones did not show any significant difference between sexes (GLM $F = 2.82$, $p = 0.96$). Males, however, had significantly wider third to fifth growth zones than females (GLM $F = 6.78$, $p = 0.01$). Another pattern was that the zone width increased with increased number of zones (GAM t -value = 20.53 , $p < 0.0001$; Fig. 6).

We observed several thinner zones within the growth zones (Fig. 2A). Nine out of 28 males displayed such secondary zones. Six out of these displayed several secondary growth zones and as many as seven was counted in one of the males. Seven males had a secondary zone between the first and second growth zone. Six out of the 23 females displayed such secondary zones (1 and 2), whereas two individuals had several. The first secondary zone was observed in the region before the first growth zone in females.

DISCUSSION

The observed zones are unlikely sectioning artifacts. They were found parallel to, and following the same curves as the eyestalks. Saw marks could not randomly have followed the curves of the eyestalks for all the samples processed during the investigation. We therefore hypothesize that the observed zones represent annual growth zones as they are consistent with previous assumptions on the life cycle of the Antarctic krill. Analysis of length frequencies and lipofuscin accumulation indicate that krill become sexually mature by their third summer and have a maximum life span of 6-7 years (Ettershank, 1983; Rosenberg et al., 1986). Growth bands can be deposited annually or on shorter time intervals (e.g., Campana and Neilson, 1982;

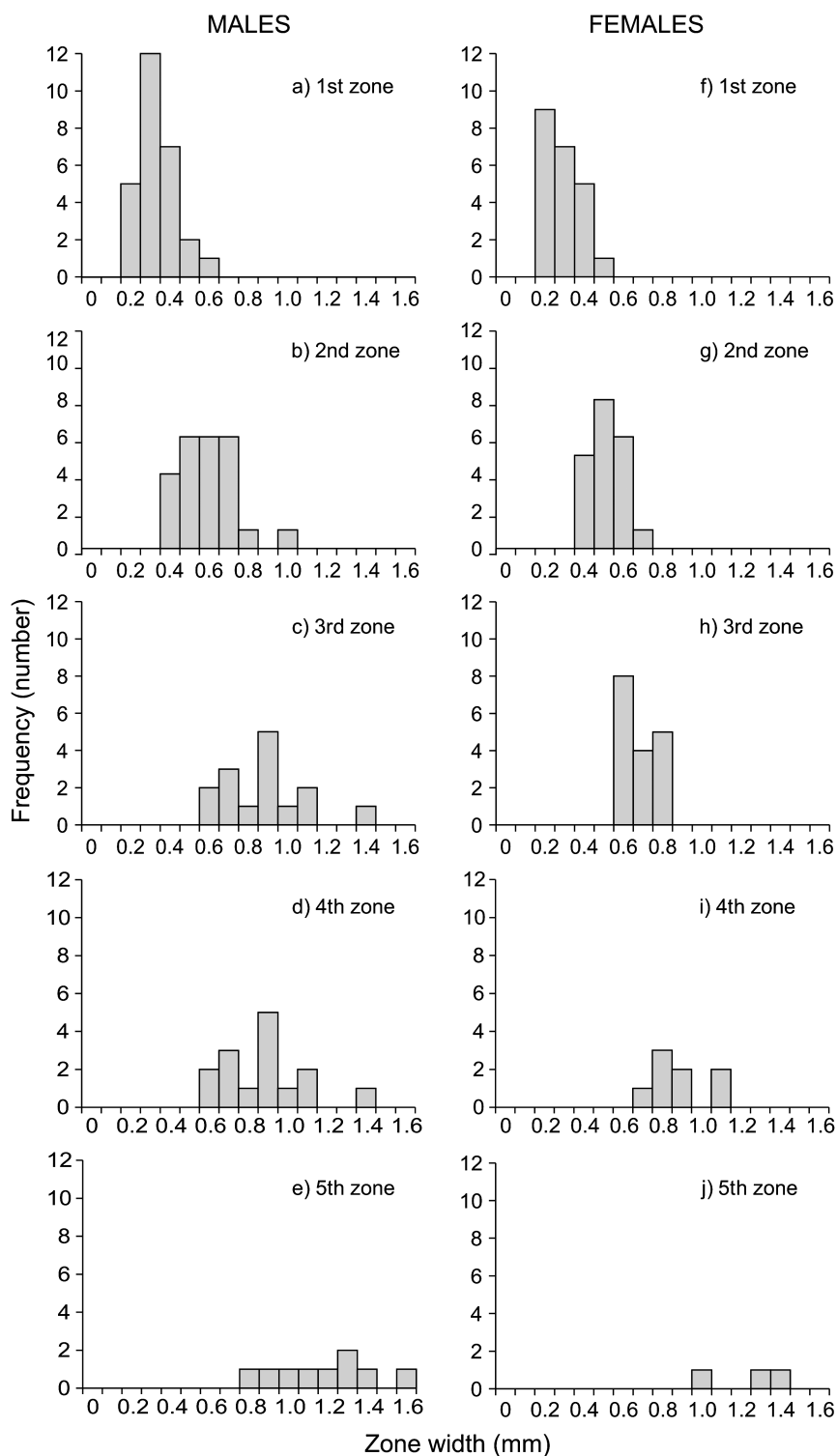


Fig. 6. Frequency of measured width of the 1st, 2nd, 3rd, 4th, and 5th zones in eyestalks of males and females of the Antarctic krill *Euphausia superba* (measurements made by one reader).

Schmitt, 1984; Iglesias et al., 1997). The Antarctic krill nevertheless inhabits cold water regions (Atkinson et al., 2006; Tarling et al., 2006), with defined annual physical and biological events, fat for instance accumulate mainly during the short summer season (Quetin and Ross, 2001).

It therefore seems likely that they could develop defined annual growth zones.

The females tended to have narrower growth zones from the third zone onwards compared with males. Information on the reproduction of the male Antarctic krill is rare, but it

has been assumed that the energetic costs are insignificant (Clarke and Morris, 1983; Miller and Hampton, 1990). The major energetic cost of reproduction in the female Antarctic krill is the accumulation of the large, lipid-rich yolk egg mass (Clarke and Morris, 1983; Nicol et al., 1995). Ettershank (1983) demonstrated that females actually reduce growth and allocate all resources into eggs. Tarling et al. (2016) also observed that the body length of females shrinks during winter, while growth stagnates in males. This considered, the observed reduction in growth after their second opaque growth zone, could indicate that the zones represent annual zones and that the females mature sexually around the age of three.

We observed several thinner zones within the growth zones, which could represent other periodic increment depositions (Fig. 2A). Such thin growth zones are also commonly observed in bivalves and fish otoliths, and Leland et al. (2011, 2015) and Kilada et al. (2012) described secondary growth zones, ossicular growth marks, or lamellae in crustaceans. These secondary growth zones could represent molting scars, but Leland et al. (2015) ruled out this possibility for the red claw crayfish (*Cherax quadricarinatus* von Martens, 1868). Adult krill mainly inhabit the productive upper water layers during summer, but can perform vertical migrations to the bottom (Schmidt et al., 2011), possibly for feeding on accumulated dead phytoplankton (see Pape et al., 2013). This strategy, exposing them to different temperatures and diet, might also influence the growth signature.

The method is suitable for detecting growth bands in the cuticle, but validation is needed before it can be used to determine age. It is our experience that the process to obtain thin and representative sections is time consuming and demands great dexterity. Alternative methods or automation of the slicing might be considered for potential use. Attempts were also made during an initial phase of the study to read sections from specimens preserved on borax-buffered formalin (4%), but it was difficult to identify growth zones in the eyestalks from these individuals. Experiments to enhance eyestalk zones could be undertaken by using different staining techniques. Before the zones can be used as an age indicator, however, legitimate challenges to this direct ageing method for crustaceans posed by Vatcher et al. (2015) needs consideration. A characterization whether the molting process in the Antarctic krill by a complete replacement of the mineralized exoskeleton, including eyestalks needs to be determined. Potentially, a cogent explanation of the potential mechanism for retention of age information in the post molted cuticle, as indicated by Kilada et al. (2012, 2015), should be provided.

If the eyestalk contains age information through molts, future studies should also try to verify the periodic incremental deposit rate. Several methods have been developed to verify age determinations from free living marine organisms (Campana, 2001), including laboratory trials using animals with known age. Such methods are often extremely time-consuming, as they require large sample sizes representing different cohorts. Caging and exposure to conditions different from their natural environment could also result in abnormal growth. A number of growth markers have been found to yield useful results in age validation and localiza-

tion of growth zones (e.g. Monaghan, 1993; Oliveira, 1996; Kilada et al., 2012; Leland et al., 2015). These consist of chemicals incorporated into the growing structures and can subsequently be used to estimate growth from time of exposure. Designing a study for verification that combines marking techniques and laboratory experiments keeping conditions similar to the krill's natural environment, as in Krafft and Krag (2015), could be a possible approach.

In conclusion, we have identified zones in the eyestalks potentially applicable as age and growth indicators for the Antarctic krill. Future work on the molting of the Antarctic krill, and verification of increment rates are prerequisite before the observed growth zones can finally be used as an age indicator. This method has the potential of providing age-based monitoring and assessment useful for management, recognizing differences in growth rates between seasons and areas in the Southern Ocean involving different environmental conditions (Siegel et al., 1990, 2002; Krafft et al., 2010, 2012, 2015).

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