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First record of cuticle bands in the stomach ossicles of the red king crab *Paralithodes camtschaticus* (Tilesius, 1815) (Decapoda: Anomura: Lithodidae) from Norway

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ABSTRACT

The red king crab *Paralithodes camtschaticus* (Tilesius, 1815) is a large predator intentionally introduced to the Barents Sea and adjacent fjords in the 1960s. Its establishment has given rise to both a high-value fishery and destructive effects on seafloor habitats and communities. Given the need for accurate information on age, growth, and longevity that could improve management and mitigation strategies for red king crab, developing and testing new aging methods for this and other crustaceans has been an active field of research. We contribute to this test bed by investigating cuticle bands in gastric mill ossicles of male and female red king crabs. Cuticle bands were detectable in most individuals studied and maximum cuticle band count was 13 for males (N = 62, 38–180 mm carapace length (CL)) and 9 for females (N = 34, size range 80–147 mm CL). There was large variation of size-at-band count and band count-at-size data. The number of cuticle bands generally increased with CL in male red king crabs; low sample size and small size range in females prevented seeing any trend. Exploring calcein staining in a sub-sample of the crabs suggested uptake of the stain, yet without a clearly defined mark, and showed deposition of ossicular material beyond the calcein stain in the subsequent year. We recommend research on the mechanism generating band deposition to shed light on how and when bands are formed as the basis for testing whether the cuticle bands may reflect chronological (specifically annual) age. Specifically, we recommend long-term maintenance of crabs, study of both moults and newly formed ossicle structures, as well as stringent testing of band periodicity with known-age crabs, including all size classes and both sexes.

Key words

Barents Sea, fisheries, gastric mill, calcein staining

INTRODUCTION

The red king crab *Paralithodes camtschaticus* (Tilesius, 1815) (Lithodidae Samouelle, 1819) is a large, generalist predator that was intentionally introduced to the southern Barents Sea and adjacent fjords in the 1960s to provide an additional food and income source (Orlov & Ivanov, 1978). Its natural distribution is across the North Pacific, including the Okhotsk, Japan and Bering seas. Red king crabs first occurred in low numbers near the location of introduction in the Russian southern Barents Sea, yet slowly spread westward, and to a limited degree offshore, through both larval dispersal and adult migration (Sundet, 2014). After the first red king crab was caught in Norwegian waters in 1977 (Orlov & Ivanov, 1978), the subsequent establishment of the species in the southern Barents Sea gave rise to a high-value commercial fishery (in Norway since 2002; Sundet & Hjelset, 2002), but also led to destructive effects on habitat and native benthic and demersal communities (Falk-Petersen *et al.*, 2011; Oug *et al*., 2011). The annual value of the quota regulated fishery (approx. US $44 million; Sundet & Hoel, 2016) is high, but so is the potential for further spreading and threats for the ecosystem outside the regulated area (Oug *et al.*, 2011, 2018; Sundet & Hoel, 2016).

Accurate estimates for critical population parameters such as age structure, age- or size-at-maturity, and mortality are needed to ensure sustainable fisheries (Enberg *et al*., 2009), rebuild depleted stocks (Kruse *et al*., 2010), or manage invasive species (Weis, 2011). The red king crab fishery in Norway is currently regulated by size (130 mm minimum carapace length (CL)) and quota for both sexes east of 26 °E, while unregulated fishing is allowed west of 26 °E to limit

68 further spreading of crabs (Sundet & Hoel, 2016). Actual chronological age can, for many
69 aquatic species, be determined directly from growth bands recorded in calcified hard structures
70 such as otoliths in fishes (Campana, 2001) and permanent shells in a variety of invertebrates
71 (Bluhm *et al*., 1998; Kilada, *et al*., 2007; Ravelo *et al.,* 2017). Until recently, similar methods
72 had not been applied to decapod crustaceans due to the loss and replacement of calcified
73 structures during moulting (Sheridan *et al.,* 2016b; Becker *et al*., 2018). Instead, indirect
74 methods, including observations of captive animals, capture-recapture experiments,
75 accumulation of lipofuscin age pigment in neural tissue, and analysis of size-frequency
76 distributions have been used to infer age (Hartnoll, 2001; Bluhm *et al*., 2001; Vogt, 2012;
77 Pinchuk *et al*., 2016). Known limitations of these approaches lead to uncertainty in the accuracy
78 of subsequent growth model estimates.

79 Given that the lack of reliable age information continues to impede assessment and
80 management of many crustacean fisheries, much research has gone into exploring the feasibility
81 of direct methods of determining age. It was recently proposed that bands discovered in the
82 endocuticle layer of stomach ossicles of decapod crustaceans may contain age information
83 (Leland *et al*., 2011; Kilada *et al*., 2012). The endocuticle is the inner part of the crustacean
84 cuticle, and underlies the exo- and subsequently the epicuticle (Vatcher *et al*., 2015). Cuticular
85 bands are recognized as paired light and dark zones in the endocuticle, and represent variations
86 in material densities observable in x-ray and transmitted light (Becker *et al.,* 2018). These bands
87 were initially described from the ossicles of the gastric mill from six crustacean species (Leland
88 *et al*., 2011; Kilada, *et al*., 2012) and the eyestalks of two additional species (Kilada *et al*., 2012).
89 These observations have since been extended to additional species of brachyuran and anomuran
90 crabs (Kilada *et al*., 2017b), crayfishes (Leland *et al.,* 2015), lobsters (Kilada *et al*., 2015),

shrimps (Kilada & Acuña, 2015), and euphausiids (Krafft *et al*., 2016). Banding in other hard structures has been linked with checks in growth, often related to seasonal food supplies, temperature cycles, or reproductive periodicity (Richardson, 2001). Research in many fish and invertebrate species over decades has established band count-chronological age relationships (Campana, 2001), which have been subsequently used in management frameworks.

Given the rather recent discovery of cuticle bands in crustacean gastric mill ossicles and eyestalks, neither the generality of the occurrence, nor their relationship to chronological age have been conclusively established. The occurrence of such bands is surprising given recent detailed studies confirming that the gastric mill, including its ossicles, is fully moulted (Vatcher *et al*., 2015; Becker *et al.*, 2018; Sheridan & O'Connor, 2018). The potential relationship of cuticle bands to age is thus uncertain. In order to add to the discussion on the putative ubiquity of cuticle bands and their interpretation, their occurrence needs to be mapped across multiple species and regions. The mechanism of their formation must also be studied, and potential links to age established, specifically the periodicity of the bands for a given species and region. If the periodicity could be established, age validation is needed and could be performed through calibration with individuals of known age (Kilada *et al*., 2012), staining with chemical markers (Leland *et al*., 2015), or other independent age estimation techniques (Campana, 2001). To contribute to this ongoing body of work and debate about the potential utility of cuticle bands as age indicators, we studied the occurrence of cuticle bands in *P. camtschaticus* from Norway, and explored the potential utility of a chemical marker for future validation studies. Finally, we compared the band counts relative to published age estimates/models for the red king crab.

MATERIAL AND METHODS

Sample collection and maintenance

Male red king crab were collected from Porsangerfjord, Norway (70.6°N, 25.6°E) in November 2014 using a combination of standard crab pots and SCUBA divers. Both females and additional males were collected from the same area in November 2015 using crab pots and frozen until further processing for band counts. Crabs collected in 2014 were transported live in a saltwater tank onboard RV *Helmer Hanssen* to the Kraknes Facility near Tromsø, and maintained in a flow-through seawater system at ambient temperature, ranging seasonally from 4–9 °C. Crabs were maintained in a 13.9 m^3 tank under a day-night cycle mimicking that of their collection site with separators keeping large, medium-sized and small crabs apart to reduce cannibalism. Crabs were fed once to twice a week *ad libitum* with Atlantic herring (*Clupea harengus* Linnaeus, 1758). Tanks were monitored daily and dead crabs were removed as soon as detected and frozen at –20 °C for later dissection. The remaining crabs were held until April 2016. Body size for all crabs was measured as carapace length (CL) from the rear of the eye orbit to the outer margin of the carapace (Donaldson & Byersdorfer, 2005) to 0.1 mm using Vernier calipers. Sex was determined using the shape of the belly flap. The holding of crabs was conducted under the regulations of and was approved by the Norwegian Animal Research Authority under Fots ID#7204.

Calcein treatment

In order to test whether calcein, a fluorescent marker for calcium carbonate, would be taken up by stomach ossicles, and whether a new band would begin to form beyond the calcein stain within a year after staining, the crabs collected in November 2014, held at the Kraknes facility, and still alive (N = 68) were stained with calcein between late January to late March 2015. We initially stained a few medium-size crabs in 125, 250, and 500 mg calcein l^{-1} seawater for 48 h to

investigate whether the calcein marker bound to gastric mill ossicles. Subsequent staining of the remaining November-collected crabs was achieved by sequentially immersing groups of crabs in a ~100 l tank of seawater containing 500 mg calcein l^{-1} seawater at ambient water temperature for 12–48 h for small and large crabs, respectively, with aeration. Stained crabs were returned to the holding tank after incubation. During or within two days after staining 18% of crabs ranging in size 34–59 mm CL died and were used, together with a few additional crabs sacrificed within a week after staining, to check if the stain was evident in the ossicles. Large (potential skip-moult) calcein-stained crabs were individually tagged around their legs with cable ties. Many crabs died during the onset of moult (possibly related to not being held individually) or otherwise over the course of the subsequent year, but their gastric mill ossicles were processed regardless for detecting cuticle bands and calcein stain. A total of 8% of crabs (N = 13) held in tanks survived until April 2016, i.e. in excess of a year after staining. Of these crabs, two tagged individuals were not observed to have moulted between staining and euthanasia; for the remaining crabs we cannot be certain.

Sample processing

Gastric-mill ossicles were obtained by dissecting the crab stomachs (Fig. 1) either after thawing frozen crabs or fresh after euthanasia. Stomachs were preserved in a mixture of glycerin, ethanol, and water (70:4:26) for at least 48 h (Kilada *et al*., 2012). The ptero-cardiac and zygo-cardiac ossicles (Fig. 1) were cleaned and embedded in cold-cure epoxy resin before preparing serial longitudinal sections (160–180 µm thickness) with a diamond-bladed Isomet saw. Sections were polished by hand using dry 0.3 µm grit lapping film, and viewed with transmitted light in 90% ethyl alcohol with a CX41 Olympus compound microscope (Olympus, Tokyo, Japan) under

40×magnification. Sections were considered of sufficient quality when cuticle bands were readable while sections were excluded when cuticle bands were poorly defined and unreadable. Digital images were taken with a DP72 Olympus video camera attached to the microscope, and images were digitally enhanced using Adobe Photoshop 12.0.4 to increase the contrast between adjacent cuticle bands. Bands were counted from the best section of each individual from the basal (adjacent to the membranous layer and hypodermis) to the distal region of the endocuticle using the first growth mark just outside the cuticular boundary between exo-and endocuticle layers as the starting point following Kilada *et al*. (2012) and Leland *et al*. (2015). Both the zygo-cardiac and ptero-cardiac ossicles were initially used for counts to investigate the clarity of the cuticle bands in the different ossicles. Because band counts were identical in the two ossicles in the subsample studied (example in Fig. 2) but were both clearer and easier to process in the ptero-cardiac ossicles, only the ptero-cardiac ossicles were processed and analyzed in the remaining crab samples.

To investigate the precision or repeatability of counting cuticle bands, band counts were made independently by two experienced readers in $N = 15$ individuals without prior knowledge of the crabs' length or of previous counts. Band count bias between readers was assessed through a bias plot where for all animals assigned a given band count by reader 1, the mean band count and 95% confidence intervals of the band count assigned by reader 2 were plotted against the reader 1 estimate (Campana, 2001). Precision estimates were calculated using the coefficient of variation (CV) following Chang (1982),

$$CVj = 100 * \frac{\sqrt{\sum_{i=1}^{R}(Xij - \bar{x})^2}}{\bar{x}\text{j}}$$

where X^{ij} is the i^{th} band count of the j^{th} crab, $\bar{x}$ is the mean band count of the j^{th} crab, and R is the number of times each crab is read. CV was averaged across all crabs sampled to produce a mean.

Fluorescence imaging of ossicle thin sections from calcein-stained crabs was conducted on a Leica TCS SP8 inverted laser-scanning confocal microscope (Leica, Wetzlar, Germany) equipped with multiple Leica HyD hybrid detectors and utilizing the Leica Application Suite Advanced Fluorescence 4.0 software. Samples were imaged using white-light laser with an excitation wavelength of 495 nm to match the excitation spectrum of the calcein fluorophore, as well as an additional excitation wavelength at 645 nm. Emission spectra were detected using one HyD detector set to 501-532 nm, the peak of the calcein emission spectrum, and natural autofluorescence was subtracted.

RESULTS

Cuticle band occurrence and counts

The ptero-cardiac ossicle sections from 96 crabs (34 females and 62 males) were of sufficient quality to discern cuticle bands. Each band was typically made up of a broad translucent zone bordered by a narrower dark band (Fig. 2), although bands were not always visible across the whole section. Cuticle bands were not readable in the ptero-cardiac ossicles from 10% of the 107 crabs which were processed. The size of male crabs with readable sections ranged 37.6–180.0 mm CL, and the number of cuticle bands ranged 2–13 for males (Fig. 3). Carapace length for females ranged 80.0–147.0 mm and the number of cuticle bands variation was 4–9.

Cuticle-band counts varied in individuals of the same size in both males and females (Fig. 3). Male crabs of e.g. ~90 mm CL had a band count between 3 and 8, whereas male crabs at ~160 mm CL showed 6–11 bands. The band count of female crabs of ~100 mm CL ranged from

4 to 9. Size-at-band-count varied by ~70 mm CL in males and by ~40 mm CL in females (Fig. 3). While we refrained from translating band count to chronological age, we overlaid cuticle band counts over published growth curves (McCaughran & Powell, 1977; Windsland *et al.*, 2013) to show where they would fall. There was a visible good agreement (Fig. 3).

The growth band counts in 15 crabs were consistent between two independent readers (Fig. 4) with a between-reader coefficient of variation (CV) of 6.8 %. Changes in technical support to the project prevented running a larger sample size.

Calcein staining

The crabs that were stained with calcein and died within two days after staining or were sacrificed within a week after staining (usable sections of 15 crabs) ranged 37.6–58.9 mm CL. The calcein mark was visible at the growing edge of thin sections in the ptero-cardiac ossicle of all individuals in this sample (example in Fig. 5C). Fluorescence was, however, also visible farther into the ossicles to varying degrees (Fig. 5C), i.e. there was not as clear a calcein mark as in similar studies of fish otoliths or bivalve shells (Campana, 2001) (Fig. 5).

The 13 crabs that survived for a year or more after calcein staining ranged 45.9–180.0 mm CL. In these crabs, the part of the ossicle with a visible calcein mark was followed by a new cuticle band (Fig. 5A), which did not show the calcein stain (Fig. 5B). This pattern was not obviously different between the two crabs that did not moult and the other eleven. This is in contrast to the more diffuse calcein mark observed at the growing edge of the ossicles of crabs sampled within a week after staining with calcein (example of a crab sacrificed three days after calcein staining in Fig. 5C).

DISCUSSION

Our study represents the first report of cuticle bands in stomach ossicles of red king crab from Norway, and is in agreement with a recent study of the same species in Alaska (Kilada *et al.*, 2017b). We revealed the presence of bands in the endocuticle of ptero-cardiac and zygo-cardiac gastric-mill ossicles, and a general increase of band counts with body size in male red king crabs. We further showed that individuals held for up to 13 months after calcein staining showed a new band beyond the calcein mark.

Presence of cuticle bands

The cuticle bands we observed in the stomach ossicles of the Norwegian red king crab were similar in appearance and location to those described in other brachyuran and anomuran crab species as well as in lobsters and crayfishes, and in the eyestalks of some shrimps and krill (full overview of taxa in Becker *et al.*, 2018). In all species where cuticle bands have been observed, including the red king crab, bands appear in the endo-cuticle as a sequence of light and dark stripes of different width, intensity, and clarity.

We confirmed the presence of cuticle bands in two types of ossicles of the gastric mill rather than just one. Cuticle bands have previously been reported from ptero-cardiac, zygo-cardiac, and meso-cardiac ossicles (reviewed by Becker *et al.*, 2018). Where multiple ossicle types were studied, investigators consistently found the same number of cuticle bands in the different ossicles within the same specimen, though clarity of bands varies from species to species and even within species. In the red king crab specifically, cuticle bands were clearest in meso-cardiac ossicles in Alaska specimens (Kilada *et al.*, 2017b), while they were more clearly visible in ptero-cardiac ossicles from Porsangerfjord individuals in the present study. High

clarity of bands from the ptero-cardiac ossicles was also found by Leland *et al.* (2015) in the red-claw crayfish.

Calcein staining

Calcein was incorporated into the stomach ossicles of *P. camtschaticus*. This is consistent with results from other recent studies, although the mark in the present study was not always as sharp as that visible in an ossicle section of a Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758). eight weeks after moult shown in Sheridan *et al.* (2016a: fig. 2C). Sheridan *et al.* (2016a) discussed calcein being resorbed during pre-moult, when the old exoskeleton is being decalcified and redeposited when new ossicles are formed, resulting in a 'recycled' mark. While we have no evidence to support this idea, we show that ossicular deposition beyond the calcein mark in red king crabs held for up to a year after staining was evident in a new zone deposited near the growing edge of the calcified structure after marking (Fig. 5). While the formation of this single new band matched the one-year time period since staining the longest held crabs, we only analysed two crabs to confirm that the crabs had not moulted during the period held in captivity, giving insufficient evidence that cuticle bands could be deposited annually and independent of moulting. After reaching sexual maturity, male red king crabs moult annually for several years before they may start skip-moulting at approximately 90 mm CL (Powell, 1967; Nilssen & Sundet, 2006). We were unfortunately not able to hold crabs for multiple years, but a multi-year experiment is essential to determine the periodicity of the cuticular bands in *P. camtschaticus*.

Annual band deposition, however, defies current understanding of ecdysis and hard structure reformation (Vatcher *et al.*, 2015; Becker *et al*., 2018, Crook *et al*., 2018; Sheridan & O'Connor, 2018). These recent studies showed that gastric ossicles were completely moulted,

which challenges the proposed direct relationship between cuticle bands and chronological age (Vatcher *et al.*, 2015; Sheridan *et al*., 2016a, b; Becker *et al*., 2018; Crook *et al*., 2018). These studies suggest cuticle bands are a result of post-moult calcification processes (Sheridan *et al*., 2016a). Becker *et al*. (2018) propose that the apparent correlation between band count and expected/known age might be explained by what they call a "secondary correlation to chronological age," whereby band count may be a function of cuticle thickness, which may increase with size and/or age. Several other studies argue for annual band deposition as a likely explanation for bands by showing that a species of Australian crayfish, *Cherax quadricarinatus* (von Martens, 1868), held for about one year after calcein treatment deposited ossicular material beyond the calcein mark that was approximately the same width as that of the previous complete cycle (Leland *et al*., 2015; Leland & Bucher, 2017). Kilada *et al.* (2012), Leland *et al.* (2015), and Leland & Bucher (2017) also argue that bands reflect chronological age and base their argument on the difference between band counts and moult frequency in the eyestalk of the northern shrimp (*Pandalus borealis* Krøyer, 1838), and in stomach ossicles of other species of decapod crustaceans. Kilada *et al*. (2017a) argued for annual band periodicity in a study on Antarctic krill (*Euphausia superba* Dana, 1850) where individuals grown from eggs hatched at two different laboratories had band deposition matching annual periodicity in eyestalks. A study on the Caribbean spiny lobster (*Panulirus argus* (Latreille, 1804)) supports their argument for interpretation of annual age band formation by showing eight to nine growth cuticular bands in the gastric mill ossicles of the same nine-year-old individuals (Gnanalingam *et al.*, 2018).

Clearly, the discrepancies in interpretation of cuticular bands among studies makes further investigation and scrutiny of the mechanisms by which cuticle bands are formed necessary. Given that it seems unlikely that some species would not moult their gastric mills, yet

294 the presence of cuticular bands has been confirmed in many species now, passing on age
295 information through moults seems less than intuitive at this stage. Histological studies before,
296 during, and after moult; validation experiments with multi-year captivity periods, including a
297 complete range of body sizes and larger sample sizes; and independent validation with
298 specimens of known ages must be conducted.
299
300 *Do band counts fit with published age information in the red king crab?*
301 Few studies have studied the age and growth of *P. camtschaticus*, and this is what motivated our
302 study. McCaughran & Powell (1977) combined mark-recapture data for males and females to
303 build a von Bertalanffy (Von Bertalanffy, 1938) growth equation (Fig. 3a) for Bering Sea red
304 king crab, and Windsland *et al*. (2013) estimated growth parameters for male red king crab from
305 tagging studies in northern Norway (Fig 3b). Growth increments and moulting probability have
306 been estimated for the Bering Sea (Vining *et al*., 2002) and Norwegian red king crab (Nilssen &
307 Sundet, 2006). While we do not have enough evidence to translate our cuticle counts into
308 confirmed chronological age, the visual overlap of the band counts with simulated probabilities
309 of ages of male red king crab by McCaughran & Powell (1977) for Bering Sea crabs and the
310 growth curve of Windsland *et al.* (2013) is obvious (Fig. 3). For example, based on moulting
311 increments, size-frequency distributions and number of probable moults (Nilssen & Sundet,
312 2006), a maximum age of 12–14 years can be expected in male crabs of 170–180 mm CL, an
313 estimate similar to our maximum band count of 13 in a 177.6 CL male crab. Age-at-size and
314 size-at-age were found to be highly variable in *P. camtschaticus* in both its native area (Stevens,
315 1990) and in northern Norway (Windsland *et al*., 2013), a pattern, again, coincident with high
316 band-at-size and size-at-band variability in our study. Is this overlap only coincidental; or

perhaps a secondary correlation as suggested by Becker *et al*. (2018)? While the recent detailed studies on ossicle moult and band structure have greatly advanced our knowledge on the subject, the exact interpretation of cuticular bands still seems unresolved.

OUTLOOK

Our study contributes to the increasing documentation of cuticle bands in decapod crustaceans, but was not sufficient to conclusively translate band counts to chronological age. Further studies should conduct an experiment where both sexes of marked, known-age crabs across the entire size range are held for several years, during which moults are documented and compared to band counts. Additional studies describing the physiological process and morphological establishment of band formation will help resolve the contradiction between seemingly periodic band formation and the loss of ossicles during moulting. Such work is a necessary prerequisite before cuticle bands could be applied as chronological age markers and be used to inform a long-term management plan of the red king crab in northern Norwegian waters.

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495 **Figure captions**

Figure 1. Ossicles in the gastric mill of the red king crab *Paralithodes camtschaticus*. Ossicles marked 4 and 5 were investigated. This figure is available in colour at *Journal of Crustacean Biology* online.

Figure 2. Thin section (180 µm) of the gastric mill ossicles of the red king crab *Paralithodes camtschaticus*. Zygo-cardiac ossicle (**A**). and ptero-cardiac ossicle (**B**) of the same individual. Red dots indicate the cuticle bands. Scale bars are 100 µm. This figure is available in colour at *Journal of Crustacean Biology* online.

Figure 3. Size-at- ossicular band count relationship of male (blue dots) and female (red dots) red king crabs (*Paralithodes camtschaticus*) with the growth curve published in McCaughran & Powell (1977) (**A**) and in Windsland *et al*. (2013) (**B**). Red and blue lines indicate female and male models, respectively. This figure is available in colour at *Journal of Crustacean Biology* online.

Figure 4. Bias plot for band count of reader 1 and 2 who counted the cuticle bands in thin sections of zygo-cardiac ossicles of the red king crab *Paralithodes camtschaticus*. Each error bar represents the 95% confidence interval about the mean count assigned by reader 2 to all crabs assigned a given band count by reader 1. The numeric values indicate the number of crabs for which cuticle bands were read at each band count group. The solid line represents one-to-one equivalence. This figure is available in colour at *Journal of Crustacean Biology* online.

Figure 5. Image of thin section (180 µm) in the zygo-cardiac of a red king crab that had moulted during the holding period and was sampled over a year after staining with calcein. Bright field light with dots indicating the cuticle bands (**A**). Fluorescent light under confocal microscope of the same section where the calcein mark (blue arrow) is deposited on the fourth band (band

518 before last) before the new growing edge (red arrow) at the fifth band (**B**). For comparison,
519 image of a calcein mark in a crab sampled three days after staining with calcein where the mark
520 extends to the growing edge (**C**).