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Age estimation obtained from analysis of octopus (*Octopus vulgaris* Cuvier, 1797) beaks: improvements and comparisons

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ABSTRACT

Two methods are currently available for age estimation in octopus beaks. They have been applied to the same specimen from a sample of 30 individuals of *Octopus vulgaris* caught in central-eastern Atlantic waters. These techniques aim at revealing growth increments in the Rostrum Sagittal Sections (RSS) and Lateral Wall Surfaces (LWS) of octopus upper and lower beaks. Both methods were improved to reduce the time of sample preparation and to enhance the appearance of the increments. For each individual, two independent readings were done for upper and lower beak sections, as well as for the lateral wall surfaces. Vertical reflected light (epifluorescence) and Image Analysis System were shown to be useful in the observation and analysis of the sequence of increments. Precision of the ageing, increment counts obtained by both techniques, and increment widths were discussed. Using upper beak RSS led to more precise age estimates, whereas preparing LWS was quicker and simpler, and revealed a higher number of increments. Therefore, our study recommends counting growth increments in LWS of beaks to age adult common octopus.

Keywords

Octopus vulgaris, age, growth, beaks, techniques

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1. Introduction

Determination of age and growth is critical to understand the life history of harvested species and to model the dynamics of their populations. Sound knowledge on life history and population dynamics is essential for assessment and management purposes. Identifying and interpreting growth increments in calcified structures (otoliths and scales of fish, statoliths of cephalopods, among other structures) produce reliable estimations of the absolute age of wild marine animals (Boyle and Rodhouse, 2005). In spite of the difficulties raised by the age determination in cephalopods, those ageing methods based on the study of incremental growth structures (Bettencourt and Guerra, 2000; Lipinski and Durholtz, 1994) are considered the most appropriate for exploited species of this group. Other available methods (Caddy, 1991) such as length frequencies are not suitable for cephalopods, since this group has high and variable growth rates, short life cycles and massive mortalities after spawning (Jereb et al., 1991; Perales-Raya, 2001; Semmens et al., 2004).

The common octopus *Octopus vulgaris* Cuvier, 1797 is one of the most important target species in the world, with catches of about 42 420 t/year for the period 2003-2007 (FAO, 2009). However, there is still not a validated and standardized age determination method for using on *O. vulgaris,* mainly due to the uselessness of statoliths for ageing species from the Octopodidae family. Recently, Doubleday et al. (2006) and Leporati et al. (2008) validated the daily deposition of increments in stylets of adults *Octopus pallidus* Hoyle, 1885 of known age. The high mortality of the paralarvae in captivity of *O. vulgaris* has not yet allowed the obtaining of known-age adults for validation purposes. However, preliminary results using chemical marking in stylets (Hermosilla et al., 2010) and beaks (Oostuizen, 2003; Perales-Raya, unpublished results) have

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shown a daily deposition of increments in adults of this species, although definitive validation is still necessary for ageing common octopus.

As beaks are present in all cephalopod species (Mangold and Bidder, 1989), any improvement in their preparation technique for ageing purposes should be useful to many commercially exploited species of this group. Beaks are composed of a chitin-protein complex (Hunt and Nixon, 1981) and secreted by a single layer of tall columnar cells, known as beccublasts that are responsible for their growth (Dilly and Nixon, 1976). The chitinization and hence growth process related to lateral walls and rostrum takes place from the rostrum tip to the wing edges (Cherel and Hobson, 2005; Miserez et al., 2008).

The beaks are structures easy to extract and manipulate. The previous freezing of the animal (samples from industrial fisheries are obtained frozen) has no effect on the visualization of the growth increments. Another advantage is that microstructures are preserved in the beak sections after being prepared according to our method. However, the possible erosion of the rostral tip during the life of the animal may bias age determination and has to be taken into account. Sections of other hard structures, such as stylets, have been recently used for octopus ageing with good results. Nevertheless, microstructure disintegration has been reported within several minutes after preparation (Doubleday et al., 2006) and the sections showed significant cracks when the animal had previously been frozen (Sousa Reis and Fernandes, 2002).

Octopus beaks have been used for ageing by Raya and Hernández-González (1998) who developed a method using sagittal sections of the rostral area. Later, Hernández-López et al. (2001) proposed a technique using the inner surfaces of lateral walls, as previously done by Clarke (1965) for *Moroteuthis ingens*.

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The aims of this study were: (1) to improve and simplify the present techniques for revealing growth increments in the beaks of the common octopus; (2) to estimate the precision of the increment counts in upper and lower beak sections and lateral wall inner surfaces; (3) to compare, for each sampled animal, the number of increments counted in the upper and lower beak sections, and in the lateral wall inner surfaces; and (4) to establish the best method for counting growth increments in the beaks of the common octopus.

2. Material and Methods

The study was carried out with a sample of 30 frozen animals from both sexes, ranging in total body weight from 90 to 5361 g (Table 1). These individuals were caught during 2007 in central east Atlantic waters (off Mauritania) by the Spanish industrial freezer trawler fleet. Once thawed, specimens were weighed and their beaks removed, cleaned and preserved in 70% ethanol. Before preparation, the beaks were rehydrated in distilled water for several days. The upper and lower beaks were weighed (mg) and the main lengths (as defined by Clarke, 1986) were obtained (mm): Hood Length (HL), Height (H), Crest Length (CL) and Rostral Length (RL).

Rostrum sagittal sections (RSS) were prepared following an improved technique based on the method developed by Raya and Hernández-González (1998) for upper and lower beaks. The rostrum area was cut with scissors and mounted in polyester resin with the lateral side facing up. After hardening of the applied resin cover, the piece was ground down with 1200 grit carborundum sandpaper. After reaching the central plane we polished with 1 µm diamond paste to obtain a smooth surface of the sagittal section.

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This section revealed a banding pattern from the rostral tip to the joining point of the hood and the crest (Fig. 1). Since the increments were visible under vertical reflected light (ultraviolet epi-illumination, if possible), it was not necessary to sand down both sides like other cephalopod hard structures such as statoliths and stylets.

Lateral wall surfaces (LWS) were prepared based on the method described by Hernández-López et al. (2001) for the upper beaks. We sagittally sectioned them with scissors to obtain two symmetrical half beaks which were cleaned by hand with water to remove any mucus attached to the inner surfaces of lateral walls. The LWS were also epi-illuminated, but here the violet light led to better results than ultraviolet one, due to the darkness of this beak zone.

The magnification chosen for RSS ranged between 200X and 400X, and we used 50X for viewing the LWS. Increments were identified and marked under the *live* camera mode (which allows for multi focal imagery), and several photos were taken to cover the whole studied area. We measured the distances between growth marks (increment width) and performed the increment count with an image analysis system (IAS, software Age&Shape). When extrapolation was necessary because increment visibility was poor (i.e. first and last portions of the anterior and posterior borders of the LWS), the IAS carried it out by using the average width of the nearest and most visible increments. To avoid tip erosion effects, the first increments located at the rostral tip of the RSS were counted in the dorsal area.

Precision is defined as the reproducibility of repeated measurements (age readings) on a given structure, whether or not those measurements are accurate (Kalish et al., 1995). The same trained reader made two repeated counts. Coefficients of Variation (CV) of the age estimates were calculated to assess precision. This method is favoured for microstructure studies as it is statistically more rigorous and thus more flexible than the use of average percent error (APE) because of the absence of an assumed proportionality between the standard deviation and the mean (Campana, 2001). For each sampled individual, we calculated the CV for the six readings: two for the upper beak, two for the lower beak, and two for the lateral walls. We obtained a total of 180 readings. For this study, CV was calculated as the ratio of the standard deviation over the mean:

$$CV = 100\% \times \sqrt{\frac{(R1 - R)^2 + (R2 - R)^2}{R}}$$

where *R*1 and *R2* were the number of increments from the first and the second reading respectively; *R* was the mean number of increments for both readings.

The normal distribution of the data was checked with the one-sample Kolmogorov-Smirnov test. Homogeneity of the variances was assessed with the Levene's test. Differences in both readings (*R1* and *R2*) for each preparation (upper and lower RSS, LWS) were compared by performing a one-way analysis of variances (ANOVA) [Zar, 1984], a Tukey's honestly significant difference (HSD) test and a Bonferroni´s multiple range post hoc test. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to a non-parametric Kruskall-Wallis test and a Games-Howell post hoc test. For all the statistical tests performed, significance level (statistically different readings) was chosen to be $P < 0.05$. The statistical analysis was performed using the SPSS package (version 9.0) from SPSS Inc.

The relationships between the number of increments and the beak measurements (HL, H, CL and RL) were calculated, as well as the relationships between the increment counts and the total body weight. Relationships calculated using the second readings (*R2)* showed the highest regression values when plotted against beak measurements.

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Besides, the second reading is supposed more reliable because of greater experience and practice.

3. Results

3.1. Methodological improvements

Although 70% ethanol was used for the preservation of the beaks during the biological sampling, our laboratory observations recommend preserving them in distilled water at a cold temperature (around 5 ºC) (Perales-Raya, unpublished results). The beaks preserved in ethanol for long time periods showed the poorest visibility of the increments, probably because ethanol dehydrates the beaks. Instead of using sections of beaks, as described by Raya and Hernández-González (1998), our cutting technique allowed the embedding of only the rostrum area, thus reducing time for grinding and polishing. Etching the section surfaces was not necessary as the ultraviolet light allowed the obtaining of more information from the deeper planes.

Vertical reflected light (ultraviolet for the sections and violet for the lateral walls) gave good results for observation of increments. Fig. 2 shows the sequence of increments in the inner surface of the lateral walls, from the anterior to the posterior edge of these structures.

In the upper and lower RSS, patterns of increments were observed from the rostrum tip to the joining point of the hood and the crest (Fig. 3A). The increments located at the rostrum tip were lost, probably due to the erosion of the rostrum during the feeding process. To avoid the tip erosion effects we usually counted the first increments in the

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dorsal area of the rostral sections, where defining a transect for counting a sequence of thin increments until the dorsal border of the hood was possible (Fig. 3B). Unfortunately, the lateral walls had no alternative reading zones, but it appeared that feeding erosion (if it exists) did not affect in the same way the readings performed in the anterior region of the lateral wall area as it did in the rostral tip of the sections.

3.2. Ageing precision, reading comparisons and growth curves

Table 1 shows the second reading values (*R2*) and Table 2 shows the results of mean CV for the three preparations of each sampled individual (upper beak RSS, lower beak RSS and LWS). RSS of the upper beak showed to be the most precise technique. Although the CV obtained were quite similar, the results showed that the less precise readings were performed in the lateral walls.

Significant differences were found in the number of increments between readings of LWS and upper beak RSS both in repeated readings *R1* (df = 89, F = 7.37, $P = 0.001$) and *R2* (df = 89, F = 6.91, $P = 0.002$), according to ANOVA and HSD Tukey post-hoc test, with a mean difference of 38 increments more in the LWS with respect to upper RSS. However, HSD Tukey test did not show significant differences between lower and upper RSS ($P = 0.055$ for *R1*, and $P = 0.123$ for *R2*). Even if HSD Tukey test did not find significant differences between lower RSS and LWS ($P = 0.315$ for *R1*, and $P = 0.198$ for *R2*), a mean difference of 16 increments more was observed in the LWS.

Fig. 4 shows the relationship between the total body weight of the sampled individual and the number of increments counted in RSS and LWS. For the same weight, a higher number of increments was counted in LWS (formula in the figure). Upper beak RSS produced the lowest counts. Regression values were: $Y = 27.978X^{0.249}$

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(r^2 = 0.75) for the LWS; $Y = 26.277X^{0.241}$ (r^2 = 0.49) for the lower beak RSS; $Y = 31.395X^{0.200}$ (r^2 = 0.54) for the upper beak RSS. Poor relationships were observed between the number of increments in lower beak RSS and the total body weight for animals over 2 000 g, and between upper beak RSS and total body weight for animals over 3 000 g.

Fig. 5A shows the results of the beak growth. The best regression (power model; *R2* = 0.76) was obtained plotting the weight of the upper beak versus the number of increments (*R2*) in the LWS. Concerning beak measurements (Fig. 5B), the best regression fit (power model; *R2* = 0.75) was obtained for the hood length (HL) of upper beak versus the number of increments (*R2*) in the LWS.

Mean widths were calculated for each increment counted in the second reading (*R2*) of the upper beak RSS, where the highest reading precision was achieved (Fig. 6A). Mean widths were also calculated for each increment counted in the second reading (R2) of the LWS, where the highest number of rings were counted (Fig. 6B). Figure 6A shows that the approximately first 50 increments (counted in the dorsal area of the RSS) were much thinner than rest of the growth marks (counted along the main axis of the RSS). This figure also shows a constant decreasing trend until approximately increment number 180, being highly scattered afterwards. Figure 6B showed a more constant trend in the mean distances of each increment in the LWS, the values being mostly comprised between 75 and 100 microns. Also here, dispersion increased from increment 180 onwards.

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4. Discussion and conclusions

Upper and lower beak RSS produced similar readings in terms of increment numbers, although the upper beak showed to give more precise age estimates. Readings performed in the LWS produced higher increment numbers than the readings in RSS (average of 38 increments more). In spite of the lower precision of the age readings in the LWS, this technique showed to be the simplest and quickest one. Those differences could be due to the fact that there were more increments to count in the LWS than in the upper and lower RSS.

Preliminary laboratory results of validation obtained so far indicate that increments seem to be laid down on a daily basis (Oosthuizen, 2003; Perales-Raya, unpublished results) in both of the studied octopus beak zones. For octopus paralarvae, increments have been shown to deposit daily on the lateral walls (Hernández-López et al., 2001).

Two hypothesis are suggested to explain the viewing of more increments in the LWS of the beak: (i) feeding erosion of the rostral tip, and even in the dorsal-posterior area of the hood (where first increments were counted), could have biased increment count toward underestimation; or (ii) increment number is underestimated in the RSS because growth marks start depositing in the rostrum several weeks after hatching. As the feeding erosion is greater in the anterior region of the beak and we performed the increment counts in the dorsal edge of the hood (where growth marks were identifiable until the posterior end), the underestimation would be negligible. At hatching, the buccal mass is fully formed and functional (Nixon and Mangold, 1996), but maybe at this stage, when the beaks are transparent and oral denticles are present in both upper and lower jaws of the paralarvae (Villanueva and Norman, 2008), the formation of internal increments inside the rostrum has not yet started.

When looking at the average widhts of the increments, upper beak RSS showed a general decreasing trend for the increments counted along the central axis of the RSS

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starting at approximately increment 90. As for this value the increment width is the widest, we can think that the fastest growth corresponds to the age of about 90 days. The thin increments counted in the dorsal area of the RSS showed an increasing trend from the edge to approximately increment 50, even if this increasing trend was not comparable to those of the increments counted along the central axis. From about increment 180, the points were highly scattered. This fact could be due to the lower number of available samples with more than 180 increments for calculating the average widths, and to the higher variability of widths observed in the posterior edge of the counting area. The trend of the average increment width observed in the LWS preparations seems to reflect the probable more constant growth of those beak surfaces. Values were also more scattered from increment 180 onwards.

Considering all the facts presented and discussed in this study, we recommend using the LWS to perform growth increment counts in the beaks of common octopus. Even if the readings were less precise than those performed in the RSS, the method is simpler and quicker. In addition, LWS are less eroded during the life of the octopus, thus avoiding the eventual underestimation problems. When daily deposition of those increments will definitively be validated for common octopus beaks, counting the growth marks of the lateral walls appears as the most suitable ageing technique for *Octopus vulgaris*.

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Fig. 1. Drawing of upper beak sagittal section. Reading area inside the left circle, where it is shown the rostral section and the increments.

Fig. 2. Increments in the inner surface of lateral walls (50X): (A) anterior region with the first increments showing with an arrow the extrapolated area; (B) medium region with increments; (C) posterior region with last increments where arrow shows the extrapolated area of the edge.

Fig. 3. (A) Appearance of increments in the central area of the beak sections (200X). (B) Dorsal region of the beak sections, where it was possible to count thin increments until the dorsal border of the hood (at the top of the image, magnification 300X)

Fig. 4. Relationship between total weight (g) and number of increments of the octopus beaks (*Octopus vulgaris*). Square: lower section, circle: lateral wall, cone: upper section. Black curve: regression for lateral walls, equation above.

Fig. 5. (A) Relationship between number of increments in the lateral wall and upper beak weight (mg) of the octopus beaks (*Octopus vulgaris*). (B) Relationship between number of increments in lateral wall and main beak measurements of upper beak. x: height, square: rostral length, cone: hood length, circle: crest length. The best regression values were obtained for the hood length and its regression line is displayed in the graph.

Fig. 6. (A) Trend of increment width in the upper sections, and (B) trend of increment width in the lateral walls of the octopus beaks (*Octopus vulgaris*).

Table 1

Sampling details for the octopus *Octopus vulgaris* beaks used in the present study. *R2*: Number of increments of the second reading.

Total weight (g)	*R2* Upper Beak	*R2* Lower Beak	*R2* Lateral Wall
91	78	78	74
532	125	120	131
537	101	92	138
605	85	107	135
647	130	101	155
708	139	167	165
724	114	143	147
900	105	108	127
925	129	160	221
1 074	159	177	168
1 106	107	117	138
1 277	112	175	176
1 315	112	127	162
1 416	128	127	202
1 526	137	172	153
1 569	149	157	173
1 741	148	205	171
1 868	140	162	196
1 879	157	173	163

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2 000	151	158	187
2 176	128	167	194
2 211	156	193	198
2 485	146	143	194
3 065	167	218	156
3 217	243	298	235
3 431	192	227	207
3 765	160	229	237
4 522	126	131	211
5 156	136	166	227
5 361	181	152	243

	Mean CV	Confidence interval (+/- 95%)	N
Rostrum Sagittal Section (Upper Beak)	3.93	1.29	30
Rostrum Sagittal Section (Lower Beak)	4.49	1.46	30
Latera Wall (Upper Beak)	4.84	1.47	30

Table 2

Precision of the two counts for Section Upper Beak, Section Lower Beak and Lateral Wall in the common octopus (*Octopus vulgaris*). CV (Coefficient of variation), N (number of samples).

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Figure 1.tif

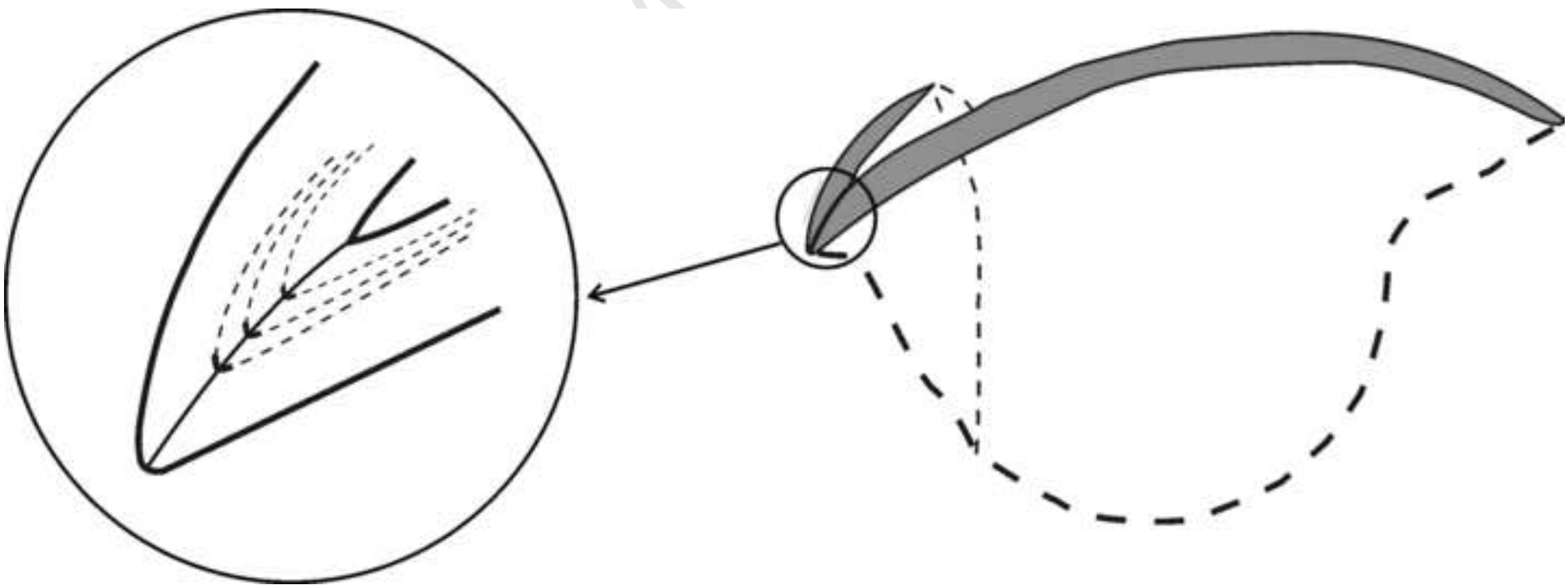

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Figure 2A.tif

Figure 2C.tif

Figure 3A.tif


20 µm

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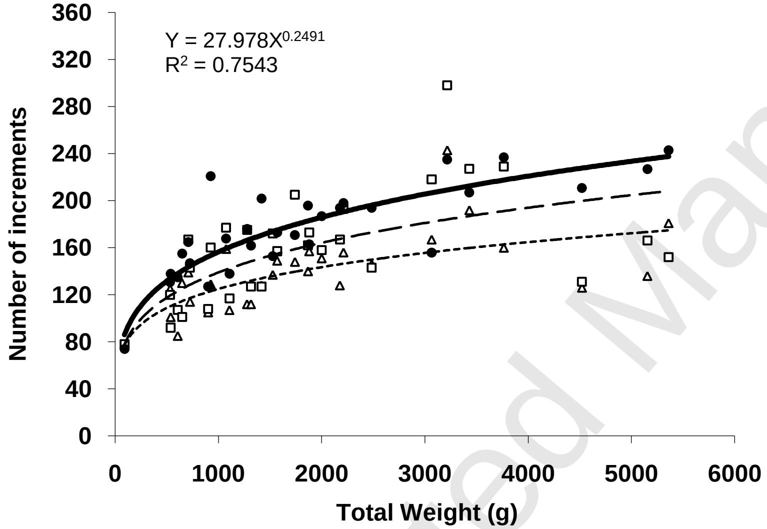

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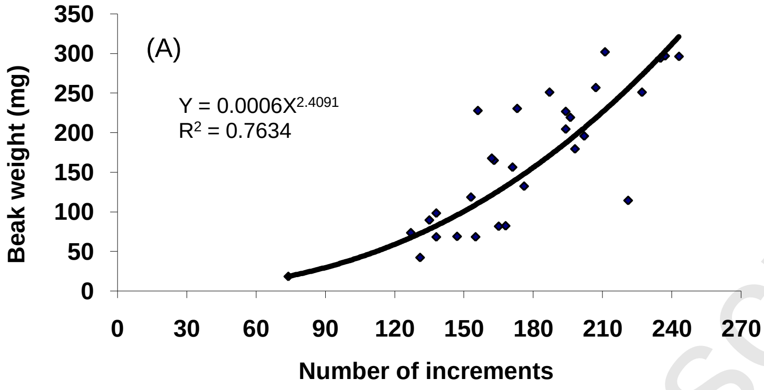

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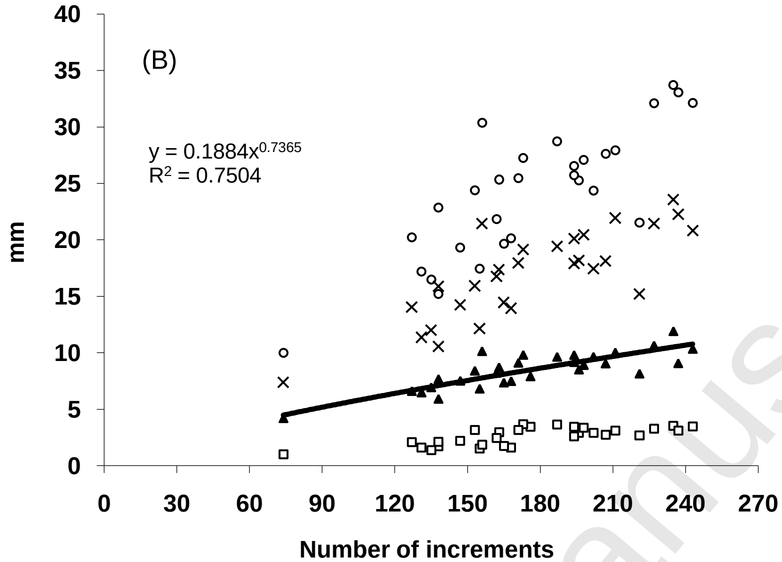

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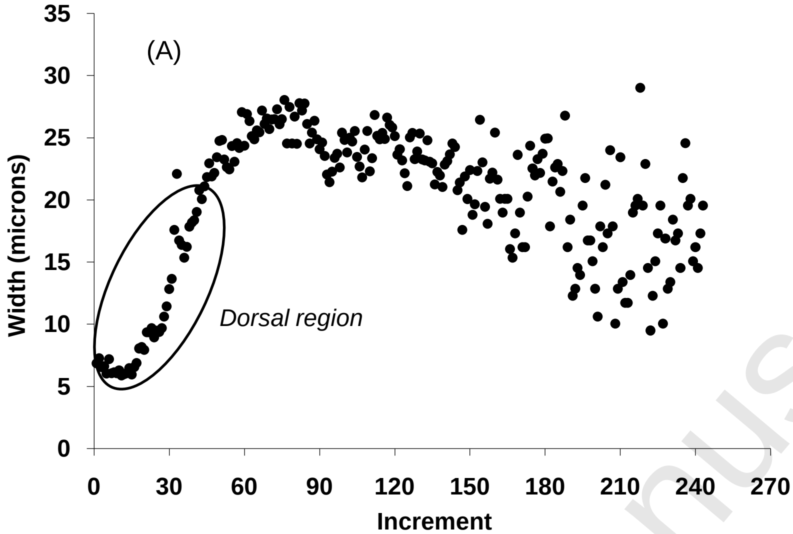

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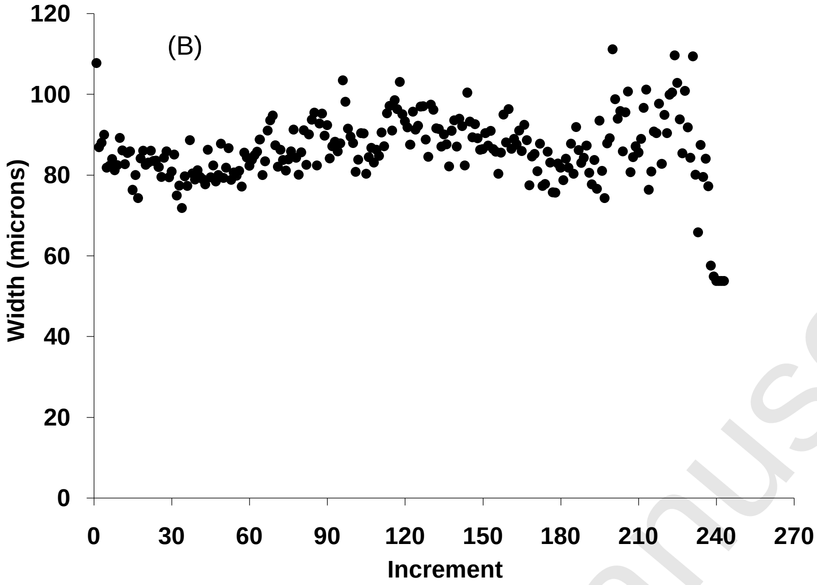

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Figure 2B.tif