Looking beyond the small: micro-CT study of eggs and development in insects: comparison of the results obtained with the Skyscan 1172 and the attachment for SEM microtomographs

Javier Alba-Tercedor1 & Isabel Sánchez Almazo2

2 Centro de Instrumentación Científica, Universidad de Granada, Campus de Fuentenueva s/n, E-18071-Granada. Spain

Aims
Eggs of insects have been studied from many different points of view and for different purposes, but so far we know, only some study exist with micro-CT (Okura et al., 2008). Thereafter, to continue with our previous research on applications of the micro-CT to biological studies (Alba-Tercedor, 2012b; Alba-Tercedor & Sánchez-Tocino 2011, 2012; Alba-Tercedor & Sáinz-Cantero, 2010, 2012; Verdú et al., 2012), and because the facility we have in our lab of a micro-CT Skyscan 1172, incited our curiosity to investigate the eggs of insects under the new perspectives that the micro-tomography offers, trying to find new lines of research and new applications. The study was done with eggs of two different orders of insects, the mayflies (Order Ephemeroptera) and lice (Order Anoplura), investigating both the external and internal structures. Moreover, recently our university acquired a Skyscan micro-CT attachment for SEM. Thereafter we decided to conduct a parallel research to compare results.

Material and Method
Mayfly eggs extracted from a preserved (in 70% ethanol) female of a mayfly from the genus Ecdyonurus (from the Sierra Nevada mountains, Southern Spain), and nits (eggs of the human lice: Pediculus humanus capitis Haeckel, 1896, Insecta: O. Anoplura) already preserved in 70% ethanol, were dehydrated and mild stained in a solution of iodine in ethanol (1% iodine metal dissolved in 100% ethanol) for 2 hours. In both cases were later submerged for 1 hour in Hexamethyldisilazane and air drying overnight (Alba-Tercedor & Sánchez-Tocino (2011 & 2012). Later were fixed in the tip of a small filament as described in Alba-Tercedor & Sáinz-Cantero (2012).

Samples were scanned with no filter, by using both, a micro-CT Skyscan 1172 C: Source Voltage = 52KV, Source Current = 85µA, and image Pixel Size = 1.4µm. Rotation step= 0.8º, Frame average=4, 360ºof Rotation; and with a Skyscan micro-CT attachment for SEM, installed on an environmental scanning electron microscope (ESEM) mod. Quanta 400 FEI. XR for the tomography were generated with an electron beam acceleration voltage of 30KV. To increase the beam current and, therefore, the XR signal, we increased the ESEM fixed last aperture from 200 µm to 500 µm, thus getting an average gain of 4; Frame average=3-5, depending on sample nature; Pixel Size = 510nm; Rotation step=0.45º until completing 180º of Rotation. Samples for the micro-CT attachment for SEM were centered by using the methodology described in Alba-Tercedor (2012a).
Figure 1: Eggs of a mayfly of the genus *Ecdyonurus*: ³CTAn shadow projections (a, b), ⁵DataViewer views of longitudinal (transaxial), and traversal (coronal) sections (obtained with a micro-CT Skyscan 1172 (Pixel size = 1.4 µm) (a, c, e)) and with the Skyscan micro-CT attachment for SEM (Pixel size = 0.51 µm) (b, d, f).
Figure 2: µCTVox volume renderings reconstructions of an egg of a mayfly of the genus *Ecdyonurus*: surface external view (a, b), longitudinal section (c), and denser chorionic structures (d). (Obtained with a micro-CT Skyscan 1172, *Pixel size* = 1.4 µm).
Figure 3: CTVox volume renderings reconstructions of an egg of a mayfly of the genus *Ecdyonurus*, obtained with a micro-CT Skyscan 1172 (*Pixel size* = 1.4 µm): surface external view (a, b), longitudinal section (c), and denser chorionic structures (d). (Obtained with the Skyscan micro-CT attachment for SEM, *Pixel size* = 0.51 µm).
CTVox volume renderings reconstructions of the external chorionic surface of an egg of a mayfly of the genus *Ecdyonurus*, permitting to compare the effect of resolution obtained both with a micro-CT Skyscan 1172 (*Pixel size* = 1.4 µm) (a), and with the Skyscan micro-CT attachment for SEM (*Pixel size* = 0.51 µm) (b).
Figure 5: CTVox volume renderings reconstructions of the egg (nit) of a human lice (a to d), and detail of the developing embryo inside (e). Scanned with a micro-CT Skyscan 1172, Pixel size = 1.4 µm)
Figure 6: CTVox volume renderings reconstructions in an apical opecular view of the egg (nit) of a human lice, scanned with a micro-CT Skyscan 1172, *Pixel size = 1.4 µm* (a) and a detail of the opecular structure of another specimen scanned with the Skyscan micro-CT attachment for SEM, *Pixel size = 0.51 µm* (b).
The Bruker-Skyscan free software (®NRecon, ®CTN, ®DataViewer, and ®CTvox) was used to reconstruct and process images, permitting to reconstruct and to get virtual slices and volume rendering reconstructions. During reconstruction with ®NRecon, besides the normal tuning procedure, it was performed x/y alignments, both an “x/y iterative” an a “x/y alignment with a reference scan”. By changing the transfer functions (obtaining consecutive overlapping symmetrical curves of the channels for the red, blue and green colors), and depleting the “opacity” curve within the ®CTvox software it was possible to get the colors and to transparent the structures. To correct the position of the sample we use both, ®DataViewer, and/or ®CTVox: This facilitate to obtain totally parallel cuts of the volume renderings when using the “Clipping Box” of ®CTVox (just pressing the Ctrl+ right click with the mouse). To isolate and to extract the embryo figured in fig. 5e, we followed a similar procedure as that described as for “undress” an aquatic beetle in Alba-Tercedor & Sáinz-Cantero (2012b). ®CTvox permits to represent and photograph a scale marked on the “clipping box”. Thereafter, the sample appears inside a box with marks. In figures 2, 3 and 4a, we maintained to make an easy comparison of the actual size within the 3D perspective. This is why in the pictures appear white lines with marks, not always parallels according with the perspective.

Results

We could get quite realistic final volume rendering reconstructions with detailed images both of the external (Figs.: 2a, 2b, 2c, 3a, 3b, 3c, 4, 5a, 5b, and 6), and the internal anatomy (Figs.: 1, 2c, 3c, 5b and 5c). Images obtained scanning by the micro-CT Skyscan 1172, where goods and sharps. But when the desired magnifications to separate structures increases (for instance, for structures with size close or lower than 10 µm), then the micro-CT attachment for SEM gave better results, as can be observed by comparing the pictures obtained with both instruments (Figs.: 1-6).

Conclusions

For the study of mayfly eggs, the micro-CT attachment for SEM (Pixel size = 0.51 µm) gives more detailed sharper images than the Skyscan 1172 (Pixel size = 1.4 µm, in fact they are close to what can be obtained with the SEM. But, with the additional advantage of evidence the internal structures that can be pointed out with the micro-CT. Thus, the micro-CT revealed denser (harder) chorionic structures on the studied mayfly’s eggs, clearly conspicuous, with both systems (Figs.: 2d & 3d). It could suggest a new line of research to test if animals living in faster running waters possess more of those structures and/or and imbricated in a different way that those living in gentle or standing waters, and an unknown adaptation. Moreover both ®CTvox volume rendering (Figs.:2 to 6) and ®DataViewer sections (Fig. 1) evidenced the presence of isolated cellular structures (blastomeric). So clearly, it could it be possible to investigate the embryonic developments of insects since the early beginning. In this respects, the possibility that software facilitate isolating and “virtually” taking out of the egg shell the developing embryo and visualize it many different perspective will represent a new dimension to the development of new embryological studies.

With respect the software, we would recommend to continue working with NRecon to implement another utility permitting to join two or more separate scans. The main problems of this instrument are, firstly to center properly the sample, and to scan with the highest magnification (lowest pixel size) an entire sample (for instance see as in figure 6b only part of the nit fitted in the screen). This is not a problem with the Skyscan 1172, because when...
sample is longer and/or wider that the size of the screen capture, it can be conducted an oversize scan: scanning separately different vertical segments and on each the left and a right half ("Camera Offset"). For the reconstruction NRecon may compose the images of the different segments and left/right sides, reconstructing the sample as a whole. Thereafter, why don’t implement some similar, but composing different scans (of up/bottom and left/right halves of the same sample?). Thus, it would permit to get scanned as a whole what in fact has been scanned in different parts.

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