Using micro-CT to elucidate the pupal case architecture as a survival strategy of a caddisfly

Javier Alba-Tercedor, Marta Sáinz-Bariáin & Carmen Zamora-Muñoz

Department of Zoology. Faculty of Sciences. University of Granada. Campus de Fuentenueva, 18071-Granada. Spain.

Aims

Larvae of caddisfly insects have been living into freshwater for some 200 million years. From an evolutionary point of view they are close related to the Lepidopteran, and as the caterpillars, the caddis larvae segregate silk. They use it to aggregate different element of substrata to build protective cases. After the larval period, the pupation for complete under water the metamorphosis imposes on them the problem of sealing themselves off for extended periods in locations where they are vulnerable to predators, parasitoids, and environmental changes (see Wiggins 2004). *Annitella amelia* Sipahiler, 1998, an European scarce caddisfly species (Trichoptera: Limnephilidae), considered to distribute only in Portugal was recently recorded in Galician region in Spain (Sáinz-Bariáin & Zamora-Muñoz, 2012). Pupal cases were collected in in a narrow spring-brook. It was observed that some of the pupa cases were built as a new tube inside an existing tubular case. Moreover it was clear that for pupation the last instar larva changes the architecture by adding internal and/or external grains of substrate. So we decided to undertake a detailed study of the case's architecture by using the micro-CT facilities existing in our laboratory. We hypothesize that the last instar larva changes the architecture of the case by adding substrate elements to assure that each half has a similar weight. Increasing, in this way, the chance that the case lye horizontally. This would represent a survival strategy by increasing the probability that the insect remains submerged into the water the time necessary to accomplish the development, and until the adult can emerges and flies.

Method

Six pupal cases from the specimens of *A. amelia* collected in a previous study (Sáinz-Bariáin & Zamora-Muñoz, 2012) were scanned with the micro-CT SkyScan 1172 C (with a. 0.5mm aluminum filter, Source Voltage = 64KV, Source Current = 100µA, and image Voxel Size = 13-15 µm. Rotation step= 0.5º, 180º of rotation scan) (Figs.1&2). The Bruker-Skyscan free software (®NRecon, ®CTan, ®DataViewer, and ® CTvox) was used to reconstruct and process images, permitting not only to reconstruct but to get virtual slices and volume rendering reconstructions (Alba-Tercedor, 2014). No stain was used.
Data set images of each case were reoriented with DataViewer, so it was possible to obtain complete horizontal/vertical longitudinal sections, and fully transversal cross-section cut slices. Finally, a new dataset, corresponding to the selected transversal cross-section new volume of interest (VOI) was saved (Fig. 3a). That new dataset was reopened with DataViewer to create a new shadow projection (these correspond to the small size figures on the top of the regular shadow projections of figures 1 and 2). After that, with the CTAn software a virtual division of each tubular case in two halves (external and internal) was performed (Fig.4: b, d), and, by running the 3D analysis plugin of CTAn, the total surface (as well the total volume) of the substrate's grains for each half was calculated (Fig. 3: c). Selecting the appropriate option of that plugin it was calculated the thickness structure, and finally by using CTVox volume rendering images were obtained, representing the substrate's grains with different colors, in accordance with its respective thickness. In a similar way as in previous papers we did before

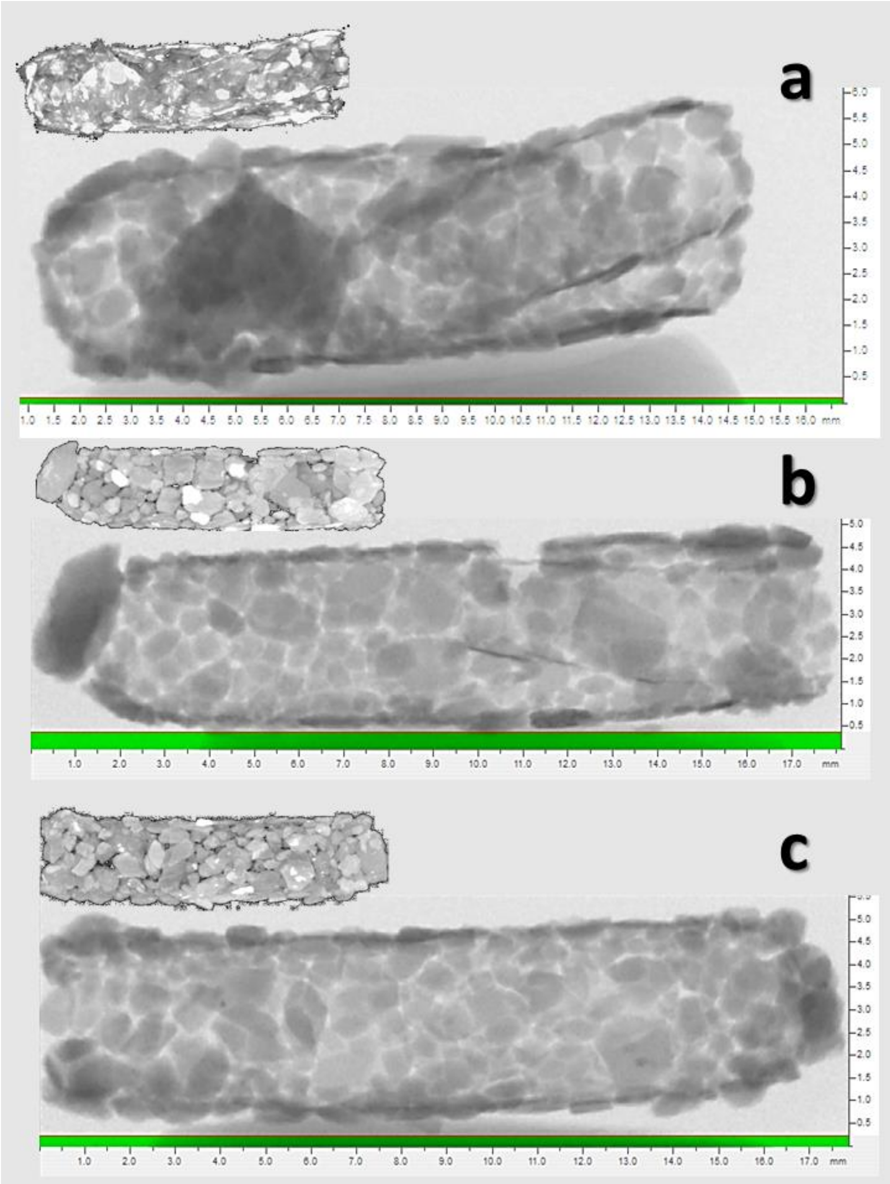

Figure 1: Shadow projections (x-ray) images of the studied caddisfly pupal cases (**a**: case #1, **b**: case #2 & **c**: case #3). On the top of each x-ray picture are new shadow projections images obtained with DataViewer once the images were reoriented and reopened (see text for details). (**a**: *Source Voltage* = 56kv, *Source Current* = 100 µA, *Pixel Size* = 13.06 µm; **b**: *Source Voltage* = 64kv, *Source Current* = 100 µA, 14.15 µm; **c**: *Source Voltage* = 64kv, *Source Current* = 100 µA, *Pixel Size* = 13.97 µm).

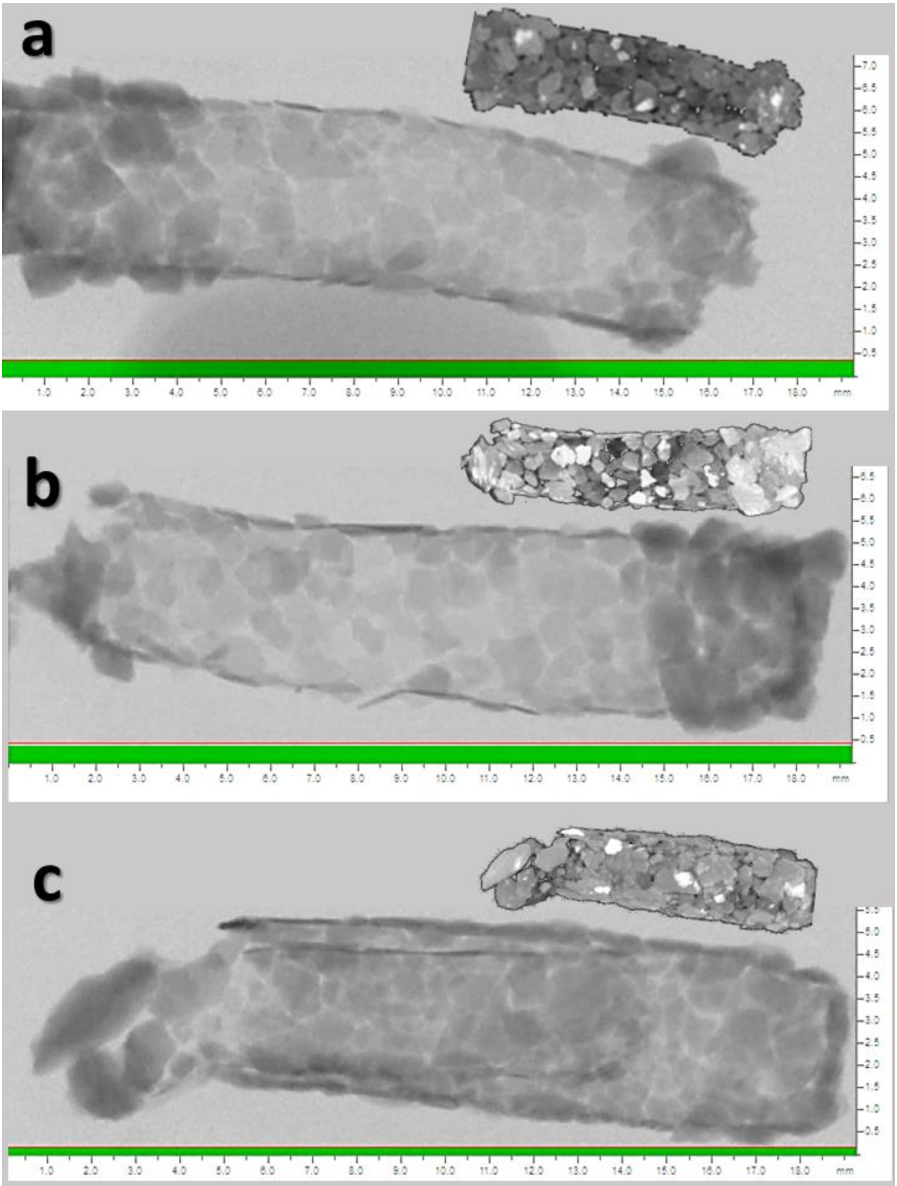

Figure 2: Shadow projections (x-ray) images of studied caddisfly pupal cases (**a**: case #4, **b**: case #5 & **c**:case #6). On the top of each x-ray picture are new shadow projections images obtained with DataViewer once the images were reoriented and reopened (see text for details). (**a, b**, **c**: *Source Voltage* = 64kv, *Source Current* = 100 µA, *Pixel Size* = 15.06 µm).

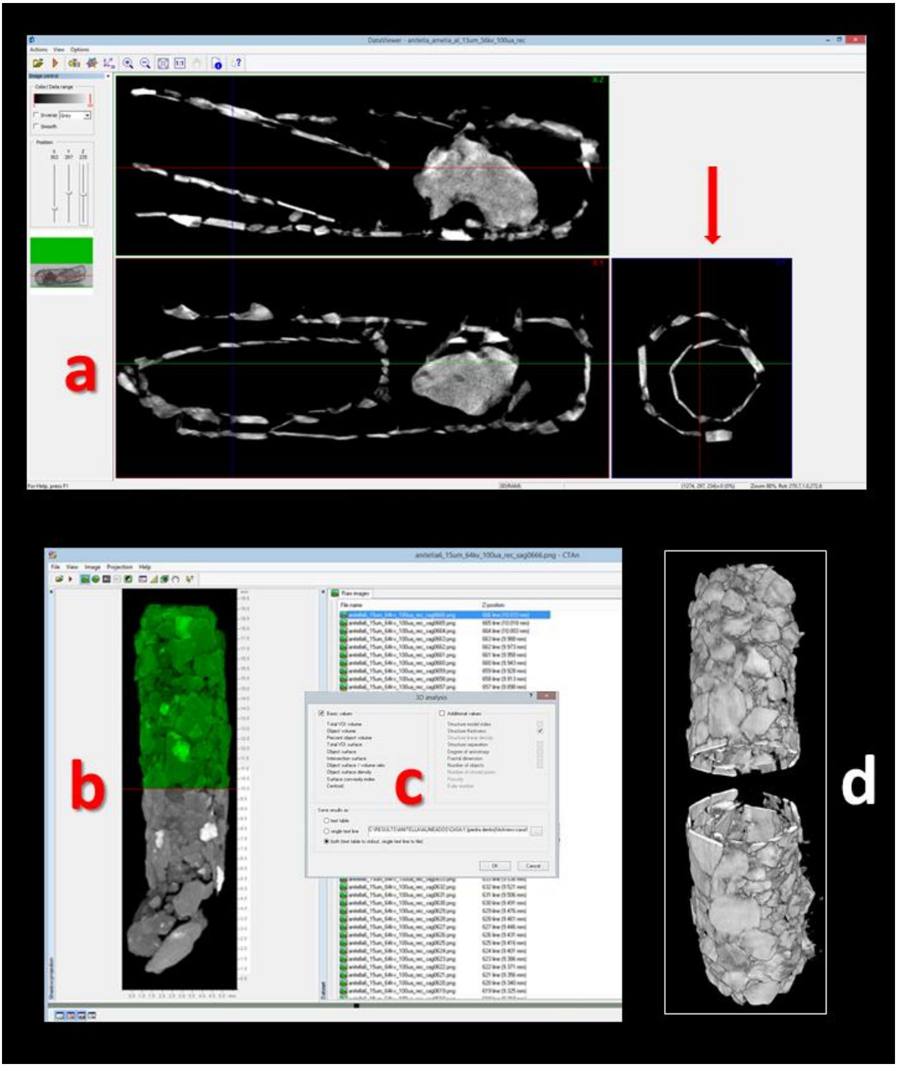

Figure 3: Data set images of each case were reoriented with DataViewer, so it was possible to obtain complete horizontal/vertical longitudinal sections, and fully transversal cross-section cuts slices (**a**). The reoriented fully transversal cross-section (indicated with a red arrow in **a**, was saved as a new volume of interest (VOI) dataset, and reopened with DAtaViewer to create a new shadow projection, the one used when opened with CTAn for analysis (**b**). A virtual division of each tubular case in two halves (external and internal) was performed (**b**, and **d**), and with the CTAn's 3D analysis plugin the total surface (as well the total volume) of the substrate's grains for each half was calculated (**c**). Observe that volume renderings of the external and internal halves represented in **d**, are just to help to understand the process, but all the calculation process of total volume and total surface of the grains from each half calculations was straight forward calculated within CTAn.

(Alba-Tercedor, Ascaso, & Wierzchos, 2014, 2015) and following the methodology detailed in Bruker-micro-CT's Method Notes (Bruker-MicroCT, 2014a, 2014b).
To test statistical differences between grains volume and surface (of external and internal case halves) non-parametric *Sign* tests (StaSoft Inc, 2005) were performed.

Results

Three cases (#1, #2 and #6) resulted doubles, with an additional tube inside (Fig. 1: a, b & Fig. 2c), the others (cases #3, #4 and #5) presented a single tube architecture (Fig. 1c & Fig. 2: a, b). In all cases conspicuous thicker rock grains are presented at both endings. Some of them resulted specially conspicuous: the big one situated inside in between the external and internal tube (see Fig. 1a, Fig. 3a, and Fig. 5: d, f, g), the "big" grain fixed on the opposite to the external opening of the tube (case #2: Fig. 1b), or the external accumulation of thicker grains observed in case #3 (Fig. 1c). Cases #4 and #5 presented accumulations of grains in both endings (Fig. 2: a, b).

To explain the above observations we stablish a starting hypothesis as follow: the architecture of the pupal case should be addressed to maintain equilibrated the weight of both halves, the "external" opening half (We), and the "internal" half (Wi) (see Fig. 6a). In the contrary either if Wi<We or Wi>We, there would be a high chance that the case remains be lying on the substrate in a vertical or close to vertical, but not horizontal position (see Fig 6b, and left cases positions on Fig. 6c). Thereafter, if the water level decreases, non-horizontal lying cases would have higher probabilities to be exposed to the air and would dry up (compare left and right situations on Fig. 6c: the case marked with and arrow would be exposed in case of a small decrease of the water level).
If the hypothesis is correct we should find a similar weight in both halves of each case, independently either if they are doubles (a new tube inside an old one) or not. So an indirect measure of the weight was performed, measuring the total surface and total volume of the whole grains substrate of each half (assuming the simplification that all grains have a similar density). Considering that logically both volume and surface are directly related to weight. Total surface (μ^2) and total volume (μ^3) of the substrate's grains from the external (with the opening) and internal halves results are summarized in Table 1. Comparisons of the total surfaces and total volumes of the external and internal halves of the pupal case are shown in Fig. 4.
When it was calculated the thickness structure of the substrate grains used to build the case #1, the CTVox's volume renderings reconstructions resulted as colored images permitting, visually, to distinguish the grains of the case in accordance with their thickness. Thus, in Fig. 5, it is clearly visible that the ticker grains are specially concentrated at both endings (Fig. 5: a, b, d & e), while the central part is constructed with thinner elements. The used elements of the new inner tube were constructed with thinner (≈lighter) grains than the surrounding ones of the external tubular case (Fig. 5f).

Discussion

When comparing the total surface of grains (both from the external parts and those from the internal parts of the pupal cases), the values resulted similar (non-statistical significance were found, $p>0.2$; although the internal half tends to be slightly heavier (with higher values of total surface) than the external half (Table 1; Fig. 4).Similar results were obtained for total volumes and external/internal halves shown non-statistical significance differences ($p>0.6$). Moreover, the internal halves tend to be slightly heavier (with higher values of total volume) than the external ones. This can be explained taking into account that once the last instar larva finish to build the architecture of the pupation case, the larva fix with silk additional grains to close the external opening. The weight of the new grains even when small must be heavy enough to equilibrate the weight of the external half. Moreover, the equilibrium should be also obtained with the weight itself of the pupa that even small should not be negligible. It is stunning, but clearly apparent the architectural behavior of the last instar larva, adding the appropriate

CASES	#1*	#2*	#3	#4	#5	#6*	Means
ExtS	35323199	24801167	27063499	23048370	21258677	32091636	27264424,667
IntS	36720348	32742492	26040551	29254573	30881087	38343592	32330440,500
ExtS/IntS	0,96	0,76	1,04	0,79	0,69	0,84	**0,85**
IntS/ExtS	1,04	1,32	0,96	1,27	1,45	1,19	**1,19**
ExtV	1.966.790	2.234.408	1.972.342	1.488.350	1.405.858	2.565.488	1.938.873
IntV	3.443.146	2.158.977	1.949.663	2.780.136	3.050.464	2.866.918	2.708.217
ExtV/IntV	0,57	1,03	1,01	0,54	0,46	0,89	**0,75**
IntV/ExtV	1,75	0,97	0,99	1,87	2,17	1,12	**1,40**

Table 1: Total surface (μ^2) and total volume (μ^3) of the substrate's grains from the external (with the opening) and internal halves, obtained with CTAn's 3D plugin (ExtS=external surface, IntS=internal surface, ExtV=external volume, and INtV=internal volume). With asterisk are marked the "double" cases See figure 3 and text for details.

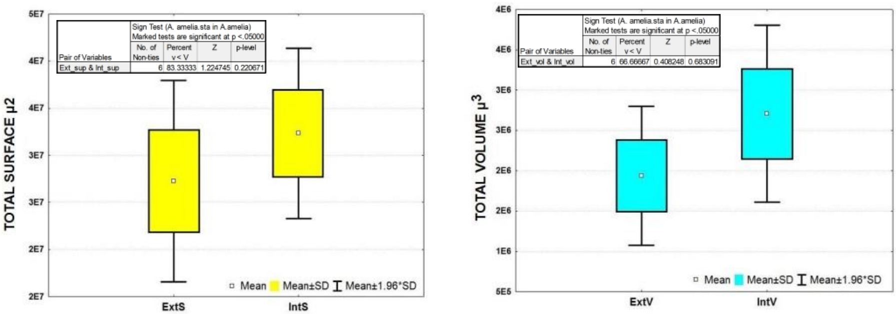

Figure 4: Box and Whisker plots of the total surfaces (left) and total volumes (right) comparison of the external (with the opening) and internal halves of the pupal case of *Annitella amelia* as indirect measures of weight. Non-statistical significances were found between both halves (p>0.2 and p>0.6). However, a clear tendency is observed in which the internal halves resulted slightly heavier (with higher values of total surfaces and total volumes).This is because in the external halves the pupa itself and some extra grains to close the case were not included in the scans (see text for details).

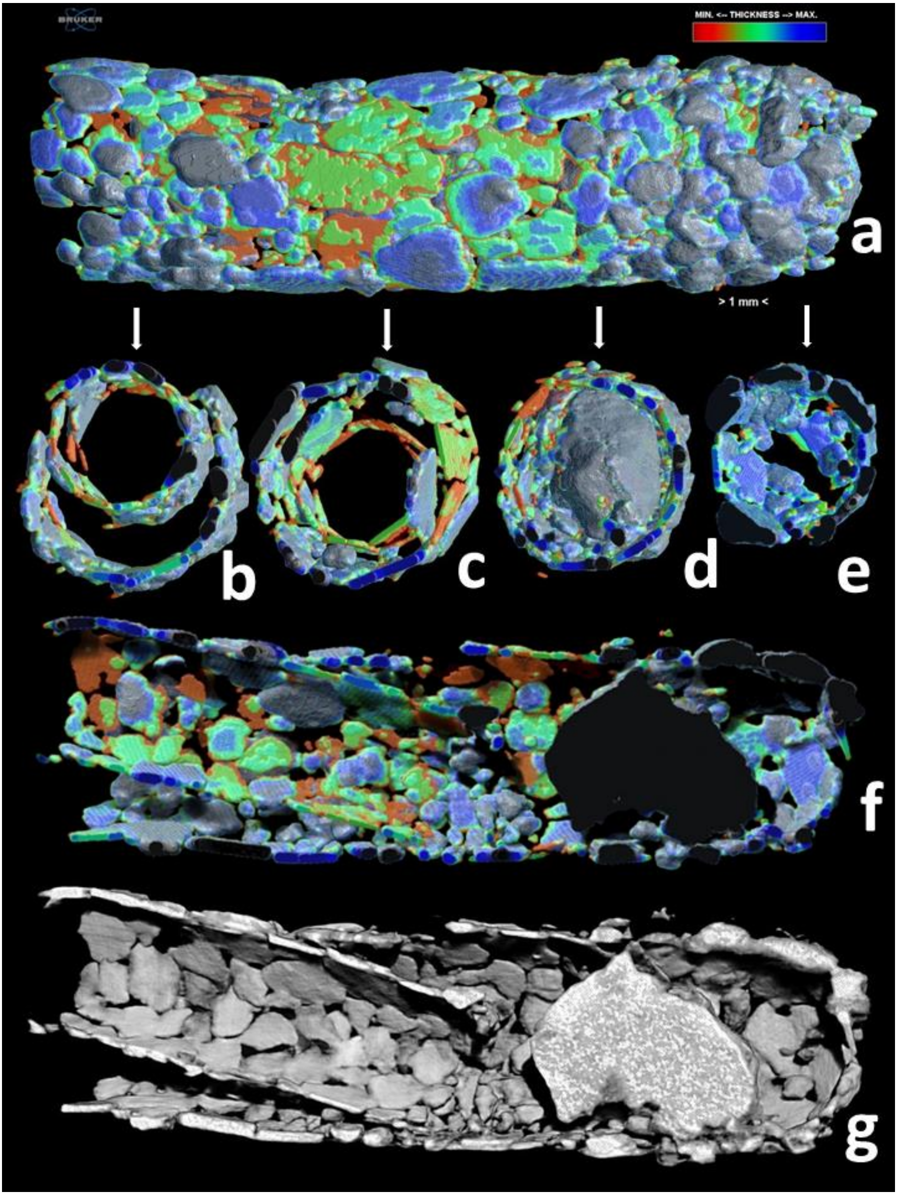

Figure 5: CTVox's volume renderings of case #1. Colors represent the thickness structure (see color bar scale at the top right): **a (**in an external view), **f** (internal longitudinal section) and **g** the same as f but rendering obtain after regular grey values images. In figures **b**, **c**, **d**, and **e**, respectively, are represented cut portions of the case corresponding to different segments (observe they are slightly left rotated to see in a perspective the inside content): **a**, anterior (external), **b** & **d**, middle and **e**, posterior (internal).

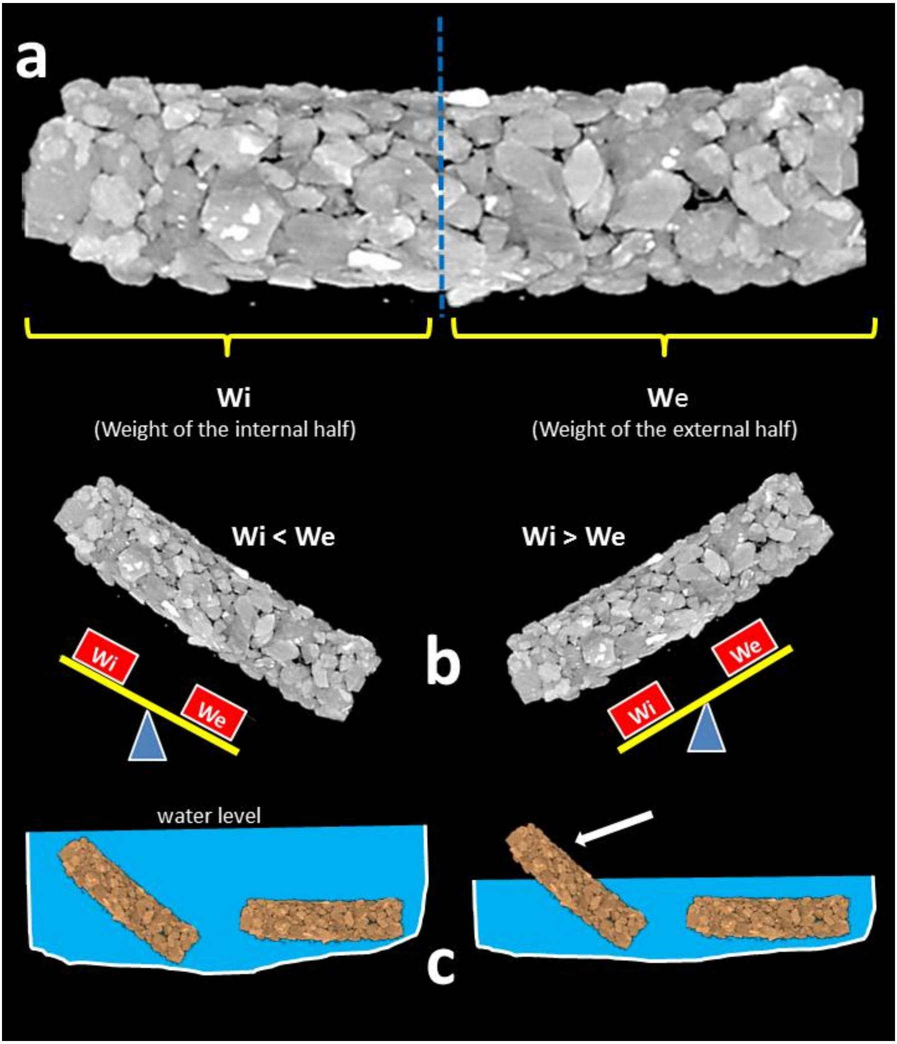

Figure 6: The starting hypothesis: the architecture of the pupal case should be addressed to maintain equilibrated the weight of both halves (**a**): the "external" opening half (We), and "internal" half (Wi). In the contrary (**b**): either if Wi<We or Wi>We, there would be a high probability that the case remains on the substrate in a position vertical or close to vertical, but not horizontal (see left cases positions on **c**). Thereafter, if the water level decreases, non-horizontal lying cases would have high probabilities of be exposed to the air and dry up (compare left and right situations on **c**: the case pointed with an arrow would be exposed in case of a small decrease of the water level).

heavier or lighter element to avoid the bias of the weight of any pupal case half, as shown in Fig. 5. It is important to point out that the observed equilibrium, securing that the weight of the case be similar in both halves, applies independently either if cases are doubles or not.
In different papers it was discuss the phylogenetic significance of building a new case for pupation, observed in some species, being the most common behavior the modification of the last instar larval case for pupation (Bohle, 2004; Malicky, 2000; G. B. Wiggins, 2001). So the presence in *A. amelia* of double cases represents to our knowledge a new finding no yet described. From this arise the question either if the external tube of these double cases represents the reutilization of an abandoned empty case from another species, or it is an addition for pupation inside the existing tube. To be able to solve this would be necessary to undertake additional experiments with live larvae.

Conclusions

The micro-CT study of the pupal cases of the caddisfly species *Annitella amelia*, permitted to point out that the larvae of the last instar, before pupation either search actively for an abandoned tubular case where they built a new tube inside, or just use their own case for pupation. In both situations they need to close the opening with new grains. This would imply an increase of weight at that end, biasing the weight (this is more apparent when a new tube inside an existing case is built). So the larva manage itself to manipulate the architecture by adding new grains in the opposite half (either outside or inside the case) to equilibrate the weight, resulting two halves of similar weight. Thereafter, the pupal case, once closed has more chance to lying horizontal on the bottom what results in and advantage avoiding to be air exposed in case of a decrease of the water level. Pupal cases were located in the shore of a narrow spring-brook at early autumn (Sáinz-Bariáin & Zamora-Muñoz, 2012). During pupation, in most caddisfly species last ca. 3 weeks, exists a high probability that fluctuations of water level occurs (this applies especially to shore sites where pupal cases were located), and hence the advantage of the observed architectural behavior adding elements to equilibrate the weight of the case in a manner that increases the chance to remain in a horizontal position on the bottom. This represents a survival strategy by increasing the probability that the insect remains submerged into the water the time necessary to accomplish the development, and until the adult can emerges and flies.

Acknowledgments

We thank Bruker-Skyscan staff for fast and effective support, their patience and effectiveness, and for their constant improvements to the software and in implementing new options we requested. But, especially, for their kindness when giving to the senior author fast and effective support to his constant pounding of queries and suggestions. In this respect, we are especially indebted to: Alexander Sasov, Stephan Boons, Xuan Liu, Phil Salmon, Jeroen Hostens, and Vladimir Kharitonov**.**
This paper supported in part by NIFA grant “Developing an Infrastructure and Product Test Pipeline to Deliver Novel Therapies for Citrus Greening Disease”, 2015. Lead Dr. S.Brown, Kansas State University & USDA.

References:

1. Alba-Tercedor, J. ,“From the sample preparation to the volume rendering images of small animals : A step by step example of a procedure to carry out the micro-CT study of the leafhopper insect k (Hemiptera : Cicadellidae)”, *Bruker Micro-CT Users Meeting 2014*, 260–288, 2014
   http://www.skyscan.be/company/UM2014/008_Javier_Alba_Tercedor.pdf

2. Alba-Tercedor, J., Ascaso, C., & Wierzchos, J., “Using micro-CT to explore pore contents in the ignimbrite, a volcanic rock in the Atacama Desert with endolithic microbial communities: the microhabitat potentially expected on Mars”, In *Bruker Micro-CT Users Meeting 2014* (pp. 47–55), 2014

3. Alba-Tercedor, J., Ascaso, C., & Wierzchos, J., "Micro-CT as a tool to identify endolithic microbial communities in pores of the ignimbrite from the Atacama Desert: the microhabitat potentially expected on Mars", *Microscopy and Analysis*, (March issue),6 pages, 2015.
4. Bohle, H. W., "Larval and pupal case building in Trichoptera: a comment to articles of G.B. Wiggins and H. Malicky in Braueria 27 and 28". *Braueria*, *31*, 9–10. 2004
5. Bruker-MicroCT, " How to make color-coded 3D models for structure thickness in CTVox Method note", *Bruker-Skyscan Method Notes*, *25*, 1–10, 2014a
6. Bruker-MicroCT, "How to make color-coded size distributions in CTVol Method note", *Bruker-Skyscan Method Notes*, 1–16, 2014b.
7. Malicky, H., "Which caddis larvae construct a new case for pupation?", *Braueria*, *27*, 19–20, 2000
8. Sáinz-Bariáin, M., & Zamora-Muñoz, C., "New record of Annitella amelia Sipahiler, 1998 (Trichoptera, Limnephilidae) in the Iberian Peninsula", *Boletín de La Asociación Española de Entomologia*, *36*(1-2), 203–205, 2012
9. StaSoft Inc., "StatSoft. *Statistica Data Analysis Software System,version 7",* 2005
10. Wiggins, G., "*Caddisflies: the underwater architect,* University of Toronto Press. Toronto, Bufalo, London, 292 pp, 2004
11. Wiggins, G. B., "Construction behavior for new pupal cases by case- making caddis larvae: Further comment. (Trichoptera: Integripalpia)", *Braueria (Lunz Am See, Austria)*, *28*, 7–9, 2001