Changing the pupal case architecture as a survival strategy in the caddisfly, *Annitella amelia* Sipahiler, 1998 (Insecta, Trichoptera)

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Abstract
Changing the pupal case architecture as a survival strategy in the caddisfly, Annitella amelia *Sipahiler, 1998 (Insecta, Trichoptera).*— In early autumn, pupal cases of the scarce caddisfly species, *Annitella amelia* Sipahiler, 1998 were collected on the shore of a narrow, shallow brook in the northwestern Iberian peninsula, in Spain. Some of the pupal cases had been built as a new tube inside an existing tubular case. Moreover, for pupation, the last instar larvae clearly changed the architecture of the cases by adding internal and/or external grains of substrate at the tips. An architectural study with micro–CT techniques made it possible to divide each case into equal halves and to indirectly measure the weight of each. As no significant differences were found, it was concluded that pupa balances its case, ensuring that it will lie horizontally on the substrate of the brook and thus avoid more vertical positions that might risk air exposure. The architectural changes could represent a survival strategy during pupation, in which the pupae remain in shallow channels ditches of small brooks.

Key words: Caddisfly, Trichoptera, Micro–CT, Pupal case architecture, Survival strategy

Resumen
Cambio de la arquitectura del estuche pupal como estrategia de supervivencia en el tricóptero, Annitella amelia *Sipahiler, 1998 (Insecta, Trichoptera).*— A principios de otoño, se recogieron estuches pupales de *Annitella amelia* Sipahiler 1998, una especie muy poco frecuente de tricóptero, en las orillas de una pequeño arroyo de cabecera situado en el noroeste del península ibérica, en España. Algunos de los estuches se habían construido como un nuevo tubo dentro de otro. Asimismo, para la pupación, la larva cambiaba la arquitectura agregando granos de sustrato en los extremos, interna o externamente. Mediante técnicas de microtomografía computerizada, se estudió la arquitectura de las construcciones y fue posible dividir cada estuche en dos mitades iguales y medir de forma indirecta el peso de cada una de ellas. Al no observarse diferencias significativas, se concluyó que las pupas equilibran el peso de las dos mitades de forma que el estuche se deposite horizontalmente en el fondo del arroyo, lo que evita el riesgo que supondría que permaneciese expuesto al aire si quedasen en una posición más vertical. Los cambios arquitectónicos podrían ser una estrategia de supervivencia durante el período de pupación, en el que las pupas permanecen en las orillas de diminutos arroyos de escasa profundidad.

Palabras clave: Tricópteros, Micro–TC, Arquitectura de estuches pupales, Estrategia de supervivencia

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Introduction

Caddisfly (Trichoptera) larvae have been living in freshwater for some 200 million years. Evolutionarily, Trichoptera are closely related to Lepidoptera. The larvae resemble caterpillars that secrete silk, which is aggregated to different elements of substrate to build protective cases. After the larval period, to carry out pupation for complete underwater metamorphosis, the insects have to sealing themselves off for long periods in locations where they are vulnerable to predators, parasitoids, and environmental changes (Wiggins, 2004). *Annitella amelia* Sipahiler, 1998, a scarce European caddisfly species (Trichoptera, Limnephilidae) considered to be distributed in Portugal only, was recently recorded in a region of Galicia in Spain (Sáinz–Bariáin & Zamora–Muñoz, 2012). Pupal cases were collected in a narrow brook. Some of the pupal cases had been built as a new tube inside an already existing tubular case. Moreover, for pupation, the last instar larva clearly changed its architecture by adding internal and/or external grains of substrate. Thus, we made a detailed study of its architecture using the micro–CT facilities in our laboratory. We hypothesize that the last instar larva changes the architecture of the case by adding substrate elements to ensure that each half has a similar weight, thereby increasing the likelihood that the case will lie horizontally. This survival strategy helps to guarantee that the pupa remains submerged in the water until the adult can emerge and fly.

Material and methods

Six pupal cases from the specimens of *A. amelia* collected in a previous study (Sáinz–Bariáin & Zamora–Muñoz, 2012) were scanned using the micro–CT SkyScan 1172 C (with a 0.5 mm aluminum filter, source voltage = 64 KV, source current = 100 µA, and image voxel size = 13–15 µm. Rotation step = 0.5º, 180º of rotation scan) (figs. 1, 2). Bruker–Skyscan free software (®NRecon, ®CTan, ®DataViewer, and ®CTvox) was used to reconstruct and process the images, enabling not only reconstruction but also virtual slicing and volume–rendering reconstructions (Alba–Tercedor, 2014). No stain was used.

Data–set images of each case were reoriented with DataViewer, providing complete horizontal/vertical longitudinal sections, and fully transversal cross–section slices. Finally, a new dataset, corresponding to the selected transversal cross–section of the new volume of interest (VOI) was saved (fig. 3A). This new dataset was reopened with DataViewer to create a new shadow projection (these being the small figures on top of the regular shadow projections of figures 1 and 2). Afterwards, using CTAn software, each tubular case was virtually divided in two halves (external and internal; figs. 4B, 4D), and by running the 3D analysis plugin of CTAn, we calculated the total surface area (as well the total volume) of substrate grains for each half (fig. 3C). We selected the appropriate option of that plugin and calculated the thickness structure. Finally, we made volume–rendering images using CTVox, representing the substrate grains with different colors according to their respective coarseness. As in previous papers (Alba–Tercedor et al., 2014), we followed the methodology detailed in Bruker–Micro–CT's Method Notes (Bruker–Micro–CT, 2014a, 2014b).

Statistical differences between grain volume and surface (of external and internal case halves) were tested using non–parametric Sign tests (StaSoft Inc, 2005).

Results

Three cases (#1, #2 and #6) were doubles, with an additional tube inside (figs. 1A, 1B, 2C), while the others (cases #3, #4 and #5) presented a single–tube architecture (figs. 1C, 2A, 2B). In all cases, conspicuous coarser rock grains appeared at both ends. Some of these grains were especially conspicuous: the large grain situated internally in between the external and internal tube (figs. 1A, 3A, 5D, 5F, 5G), the large grain fixed opposite to the external opening of the tube (case #2: fig. 1B), and in case #3, the external accumulation of visible coarser grains (fig. 1C). Cases #4 and #5 had accumulations of grains at both ends (figs. 2A, 2B).

To explain the above observations, we propose a starting hypothesis as follows: the architecture of the pupal case should maintain a balanced weight of the two halves, the 'external' opening half (We), and the 'internal' half (Wi) (fig. 6A). On the contrary, either if Wi < We or if Wi > We, the case would have a high likelihood of lying on the substrate in a vertical or close to vertical position, but not a horizontal position (see figure 6B, and left case positions in fig. 6C). Then, if the water level decreases, cases not lying horizontally would have higher probabilities of being exposed to the air and drying up (compare the left and right situations in figure 6C: the case marked with an arrow would be exposed in case of a minor decrease in the water level). Figure 7 shows the small brooks where the pupal cases were collected and the detail of the shallow ditches.

If the hypothesis were correct, we should find a similar weight in both halves of each case, regardless of whether or not they are doubles (a new tube inside an old one). Thus, the cases were indirectly weighed, measuring the total surface and total volume of the whole grains of substrate on each half (assuming the simplification that all grains have a similar density and considering that both volume and surface are directly related to weight). Table 1 summarizes the results for the total surface area (μ^2) and total volume (μ^3) of the substrate grains from the external (with the opening) and internal halves. Figure 4 shows the comparisons of the total surface areas and total volumes of the external and internal halves of the pupal case.

After calculating the thickness structure of the substrate grains used to build case #1, we observed that the volume reconstructions by CTVox rendered as colored images permitted the grains of the case to be visually distinguished according to their coarseness. Thus, figure 5 clearly shows that the coarser grains are

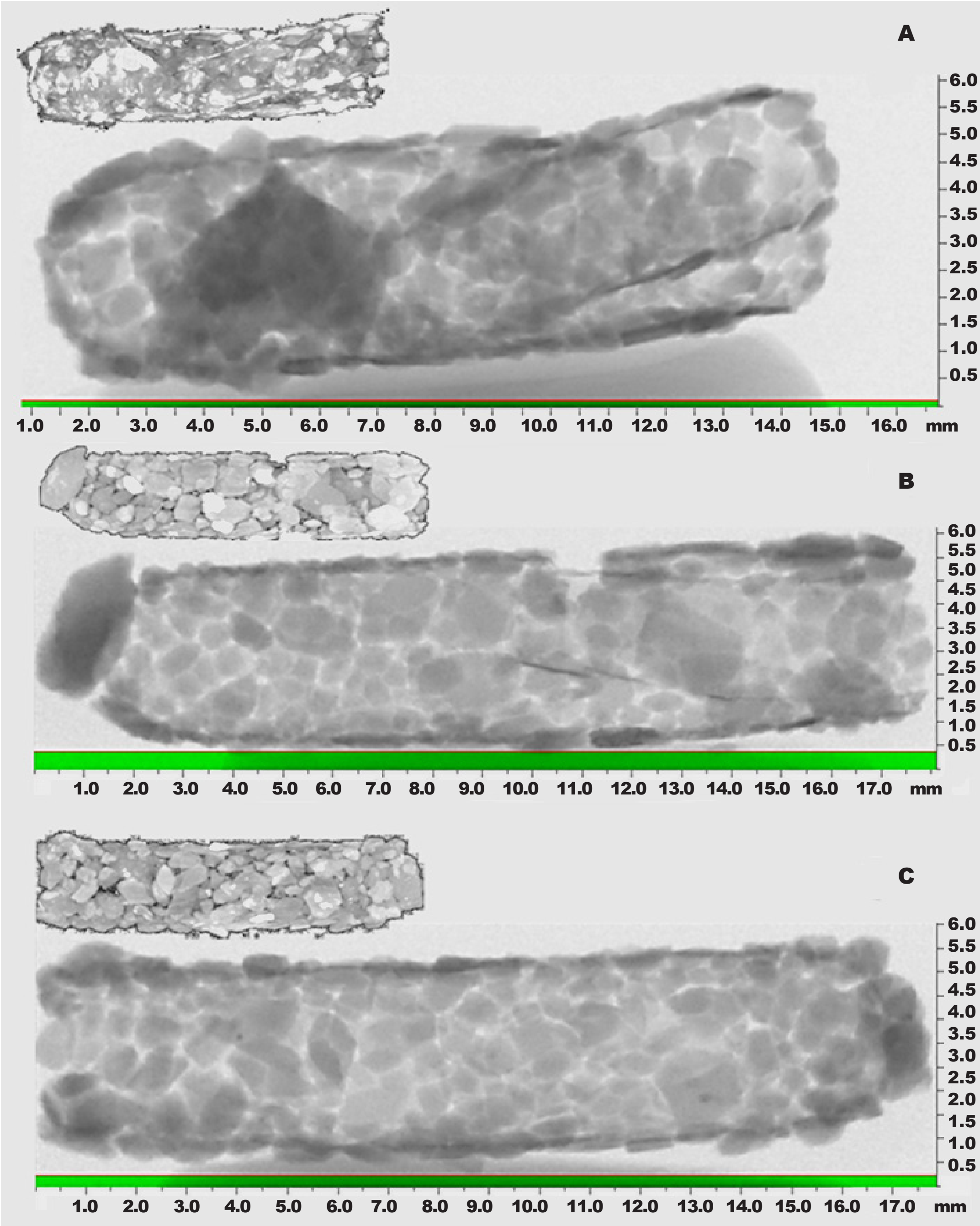

Fig. 1. Shadow projection (X–ray) images of the caddisfly pupal cases studied (A. Case #1; B. Case #2; C. Case #3). Above each X–ray image there are new shadow projections captured with DataViewer after the images were reoriented and reopened (see text for details): A. Source voltage = 56 kv, source current = 100 µA, pixel size = 13.06 µm; B. Source voltage = 64 kv, source current = 100 µA, pixel size = 14.15 µm; C. Source voltage = 64 kv, source current = 100 µA, pixel size = 13.97 µm.

Fig. 1. Imágenes de rayos X de los estuches pupales de tricópteros estudiados (A. Estuche #1; B. Estuche #2; C. Estuche #3). Encima de cada imagen de rayos X se sitúan reconstrucciones adicionales, obtenidas con el programa informático DataViewer, tras reorientar su posición (véase el texto para obtener más detalles): A. Voltaje de la fuente de alimentación = 56 kv, intensidad de la fuente de alimentación = 100 µA, tamaño de vóxel = 13,06 µm; B. Voltaje de la fuente de alimentación = 64 kv, intensidad de la fuente de alimentación = 100 µA, tamaño de vóxel = 14,15 µm; C. Voltaje de la fuente de alimentación = 64 kv, intensidad de la fuente de alimentación = 100 µA, tamaño de vóxel = 13,97 µm.

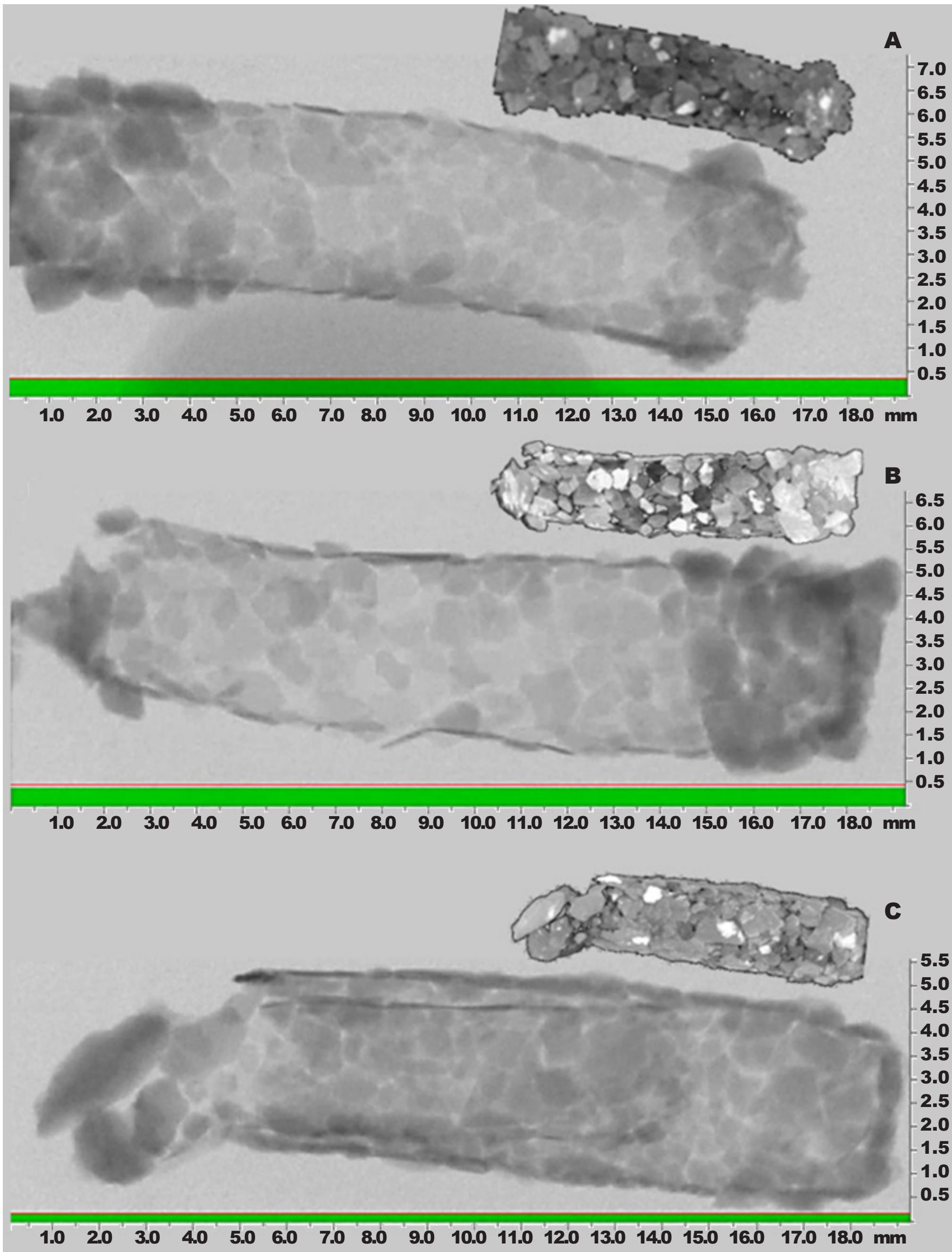

Fig. 2. Shadow projection (X–ray) images of caddisfly pupal cases studied (A. Case #4; B. Case #5; C. Case #6). At the top of each X–ray image there are new shadow projections images captured with DataViewer after the images were reoriented and reopened (see text for details): A, B, and C. Source voltage = 64 kv; source current = 100 μA; pixel size = 15.06 μm.

Fig. 2. Imágenes de rayos X de los estuches pupales de tricópteros estudiados (A. Estuche #4; B. Estuche #5; C. E estuche #6). Encima de cada imagen de rayos X se sitúan reconstrucciones adicionales obtenidas con el programa informático DataViewer, tras reorientar su posición (véase el texto para obtener más detalles): A, B y C. Voltaje de la fuente de alimentación = 64 kv; intensidad de la fuente de alimentación = 100 μA; tamaño de vóxel= 15,06 μm.

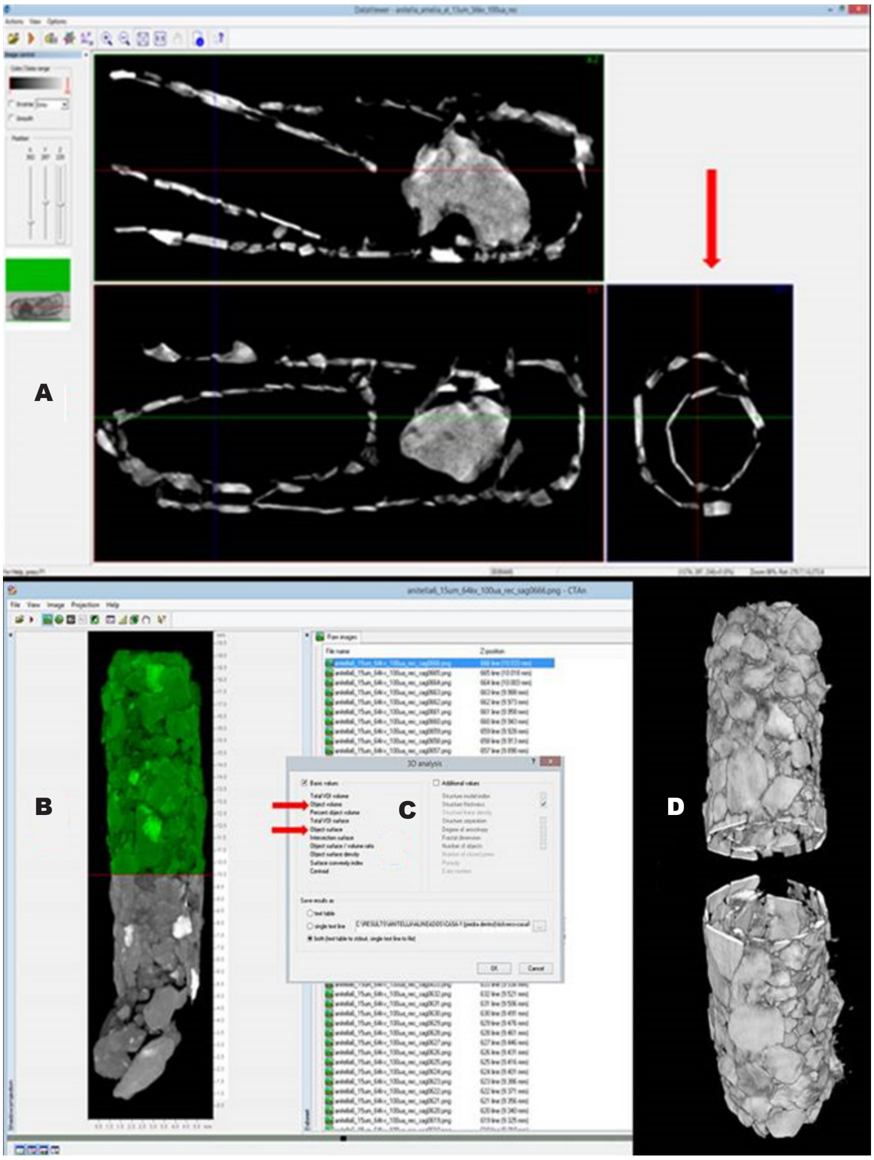

Fig. 3. Data–set images of each case were reoriented with DataViewer, making it possible to obtain complete horizontal/vertical longitudinal sections, and fully transversal cross–section slices (A). The reoriented fully transversal cross–section (indicated with a red arrow in A) was saved as a new volume of interest (VOI) data set, and reopened with DataViewer to create a new shadow projection the one used when opened with CTAn for analysis (B). Each tubular case was virtually divided into two halves (B: external and D: internal), and with the CTAn's 3D analysis plugin the total surface area (as well the total volume) of the substrate's grains for each half was calculated (C). Note that volume renderings of the external and internal halves represented in D, are only to facilitate an understanding of the process, but all the calculation process of total volume and total surface area of the grains from each half was calculated directly with CTAn.

Fig. 3. El conjunto de imágenes de cada estuche se reorientó mediante DataViewer para poder obtener secciones horizontales y verticales completas y cortes completamente transversales (A). La sesión transversal perfectamente reorientada (indicada con una flecha roja en A) se guardó como una serie de imágenes que representan un nuevo volumen de interés (VOI) que con DataViewer permitió crear nuevas imágenes y que fue usado con CTAn para el análisis (B). Cada estuche tubular se dividió virtualmente en dos mitades (B: externa y D: interna) y, mediante el complemento para análisis 3D de CTAn, se calcularon la superficie total y el volumen de los granos de substrato de cada mitad (C). Las reconstrucciones volumétricas de las mitades externas e internas, representadas en D, son simplemente para ayudar a comprender el proceso, pero todo el proceso para calcular el volumen total y la superficie total de los granos de substrato de cada mitad se realizó directamente con CTAn.

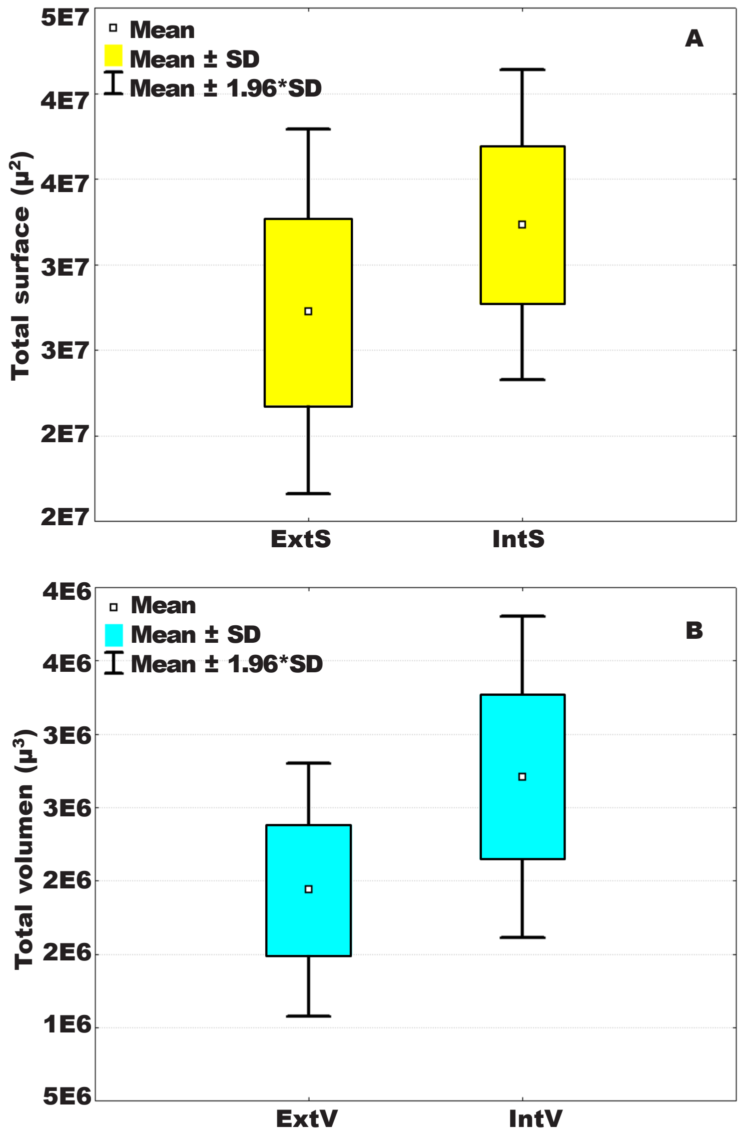

Fig. 4. Box and whisker plots comparison of the total surfaces (A) and total volumes (B) of the external (with the opening) and internal halves of the pupal case of *Annitella amelia* as indirect measures of weight. No statistical significance was found between the two halves ($p > 0.2$ and $p > 0.6$). However, the internal halves clearly tended to be 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 seal the case were not included in the scans (see text for details).

Fig. 4. Comparación mediante diagramas de cajas y "whisker plot" de la superficie total (A) y el volumen total (B) de las mitades externas (con la abertura del estuche) e internas del estuche pupal de Annitella amelia *como medidas indirectas de peso. No se encontraron diferencias estadísticas significativas entre ambas mitades (*p > 0,2 *y* p > 0,6*). Sin embargo, se observó una clara tendencia a que la mitad interna fuera ligeramente más pesada (con mayores valores de superficie total y volumen total). Esto es debido a que en la mitad externa tanto la pupa como los granos de sustrato adicionales para cerrar el estuche no se incluyeron en los escaneos (véase el texto para obtener más detalles).*

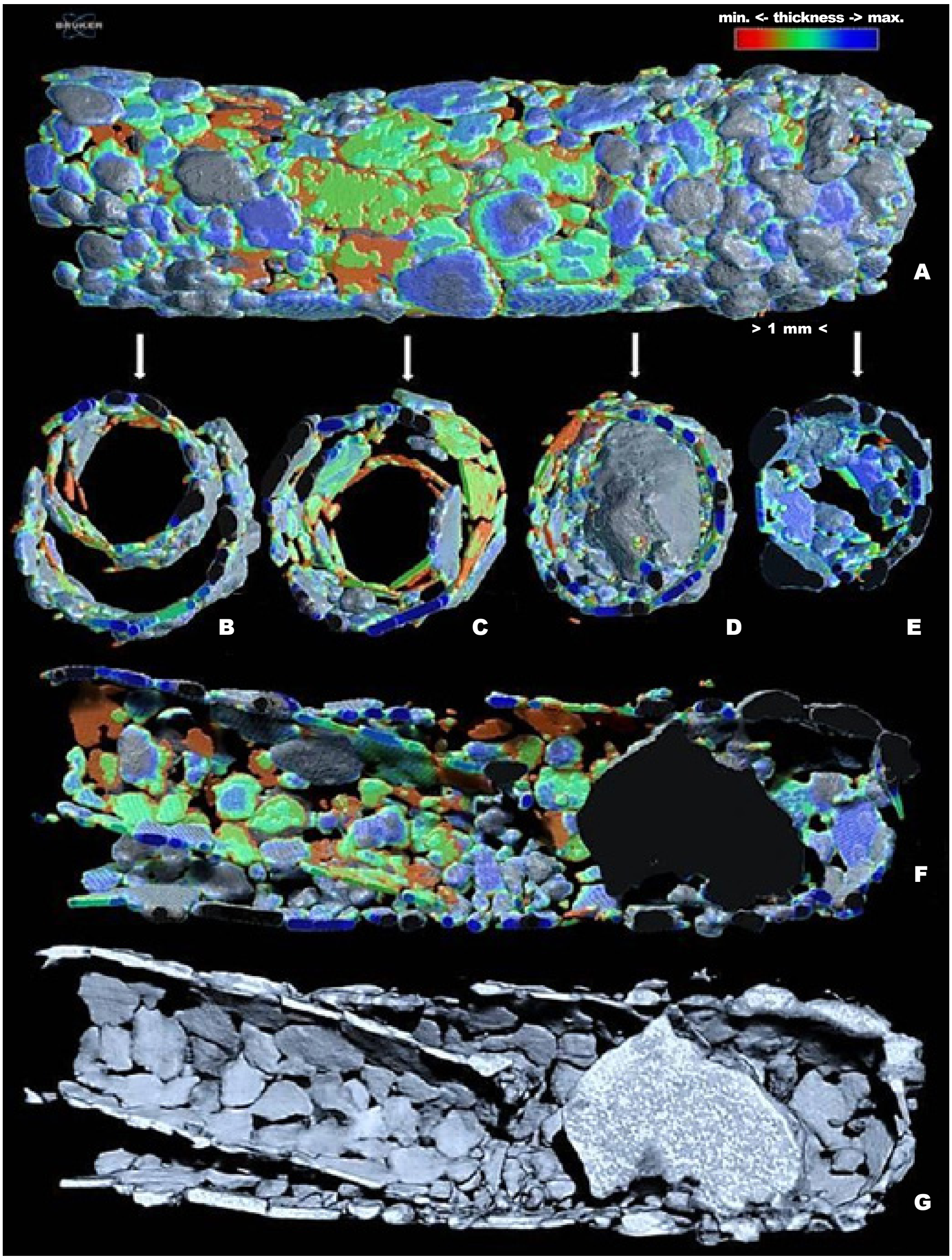

Fig. 5. CTVox volume renderings of case #1. Colors represent the thickness structure (see bar scale): A. External view, F. Internal longitudinal section; G. The same as F but the rendering was made in regular gray–value images. Figures B, C, D and E, respectively, represent cut portions of the case corresponding to different segments (note that they are slightly rotated to the left to show the inside content): A. Anterior (external); B, D. Middle; E. Posterior (internal).

Fig. 5. Reconstrucciones volumétricas del estuche #1, obtenidas con CTVox. Los colores representan el grosor de las estructuras (véase la escala): A. Vista externa; F. Sección longitudinal interna; G. Igual que F pero la reconstrucción volumétrica de la imagen se hizo tonos grises. En las figuras B, C, D y E se representan, respectivamente, los cortes del estuche a distintos niveles (obsérvese que están ligeramente rotados a la izquierda para poder ver el contenido interior): A. Anterior (exterior); B, D. Media; E. Posterior (interna).

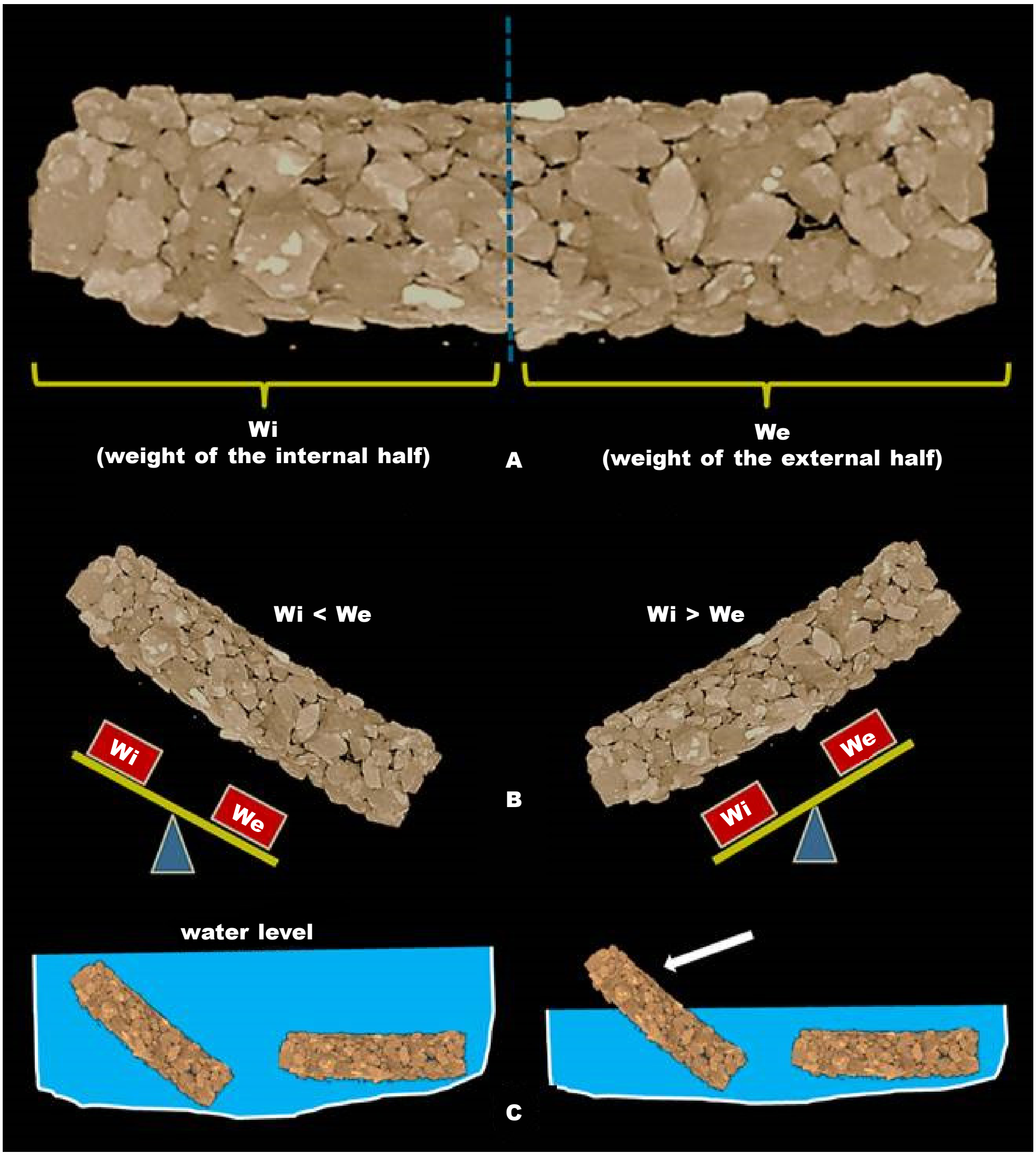

Fig. 6. The starting hypothesis: the architecture of the pupal case should maintain the weight of both halves balanced (A): the 'external' opening half (We), and 'internal' half (Wi). On the contrary (B), either if Wi < We or if Wi > We, there would be a high probability that the case on the substrate would take a vertical or close to vertical position, but not horizontal (see left case positions on C). Thereafter, if the water level descends, cases not lying horizontal would have high probabilities of exposure to the air and drying (compare left and right situations on C: the case indicated with an arrow would be exposed in case of a slight descent in the water level).

Fig. 6. La hipótesis de partida: la arquitectura del estuche pupal debería estar dirigida a mantener equilibrado el peso de las dos mitades (A): la mitad "externa" de la abertura (We) y la mitad "interna" (Wi). Por el contrario (B): tanto si Wi < We como si Wi > We, existiría una elevada probabilidad de que el estuche permaneciera en el sustrato en posición vertical o casi vertical, pero no en posición horizontal (véase la posición de los estuches a la izquierda en C). Así, si el nivel del agua desciende, los estuches que no estén en posición horizontal tendrían una gran probabilidad de quedar expuestos al aire y secarse (compárense las situaciones izquierda y derecha en C: el estuche señalado con una flecha quedaría expuesto en caso de que se produjera un leve descenso del nivel del agua).

Fig. 7. General aspect of the small brooks from where the pupal cases were collected and detail of the small and shallow ditch.

Fig. 7. Aspecto general de los pequeños arroyos donde se colectaron los estuches pupales y detalle del cauce reducido y poco profundo.

concentrated at each end (figs. 5A, 5B, 5D, 5E), while the central part is constructed with finer elements. The elements used for the new inner tube were constructed with finer (≈ lighter) grains than those surrounding the external tubular case (fig. 5F).

Discussion

When comparing the total surface area of the grains (both from the external parts and those from the internal parts of the pupal cases), the values were similar (no statistical significance, $p > 0.2$; although the internal half tended to be slightly heavier (with higher values of total surface area) than the external half (table 1, fig. 4). Similarly, for total volumes and external/internal halves, no statistically significant differences were found ($p > 0.6$). Moreover, the internal halves tended to be slightly heavier (with higher values of total volume) than the external halves. This can be explained taking into account that once the last instar larva finishes building the pupation case, the larva uses silk to fix additional grains to close the external opening. The weight of the new grains, even when small, must be heavy enough to balance the weight of the external half. Moreover, the equilibrium should also be established with the weight of the pupa itself, which although small is not negligible. The clear architectural behavior of the last instar larva is striking because it adds the appropriate heavier or lighter element to avoid any weight bias of either half of the pupal case, as shown in figure 5. It is important to point out that the observed equilibrium, ensuring that the weight of the case is similar in both halves, applies regardless of whether or not cases are double.

Typically, case–carrying caddisflies pupate in the larval case after they have fixed it to coarser material from the stream bottom and sealed off the anterior opening with a silk, perforated cover (Wiggins, 2004). This is a significant behavioral distinction of the suborder Integripalpia (most of the case–carrying caddisflies), and therefore species departing from the normal behavior are noteworthy (Wiggins, 2001). A few species of limnephilids, brachycentrids, and phrygaenids can build new cases before pupation (Malicky, 2000); several papers have discussed the phylogenetic significance of

Table 1. Total surface (μ^2) and total volume (μ^3) of the substrate grains from the external (with the opening) and internal halves, determined with CTAn's 3D plugin: ExtS. External surface; IntS. Internal surface; ExtV. External volume; IntV. Internal volum; * 'Double' cases (see figure 3 and text for details).

*Tabla 1. Superficie total (μ^2) and volumen total (μ^3) de los granos de sustrato de las mitades externas (con abertura) e internas, obtenidos mediante el complemento 3D del programa informático CTAn: ExtS. Superficie externa; IntS. Superficie interna; ExtV. Volumen externo; IntV. Volumen interno; * Estuches dobles (véase la figura 3 y el texto para obtener más detalles).*

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	1.21
ExtV	1966790	2234408	1972342	1488350	1405858	2565488	1938872.667
IntV	3443146	2158977	1949663	2780136	3050464	2866918	2708217.333
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	1.48

building a new case for pupation (Malicky, 2000; Wiggins, 2001; Bohle, 2004). Nevertheless, this behaviour is not a generalization, and intraspecific variability has been recorded (Statzner, 2011). Even if a new case is not built for pupation, the pupal case of case–carrying caddisflies may have some mineral fragments that are lacking in the larval case (Wiggins, 2004), which the larvae presumably has to find near the location where they pupate. This applies to certain goerids and odontocerids in which their larvae close the tube openings with small pieces of gravel prior to pupation. However, this behaviour has not been recorded before for limnephilids. Thus, the presence of double cases in *A. amelia* is a new finding in the literature available. This finding raises the question as to whether the external tube of these double cases represents the reuse of an abandoned empty cases from another species, or whether it is an addition for pupation inside the existing tube. The answer to this question requires additional experiments with live larvae.

Conclusions

The micro–CT study of the pupal cases of the caddisfly species *Annitella amelia* indicates that before pupation, the last–instar larvae either search actively for an abandoned tubular case where they build a new tube inside or use only their own case for pupation. In both situations, they need to seal the opening with new grains. This would imply an increase in weight at that end, biasing the overall weight (this is more apparent when a new tube inside an existing case is built). Therefore, the larva must manipulate the architecture by adding new grains to the opposite half (either outside or inside the case) to balance the weight of the two halves. Once the pupal case is closed, it has more likelihood of lying horizontally on the bottom of the brook, thus avoiding air exposure in the event of a fall in the water level. Pupal cases were located on the shore of a narrow brook in early autumn (Sáinz–Bariáin & Zamora–Muñoz, 2012). During pupation (in most caddisfly species lasting ca. three weeks) there is a high probability of fluctuations in water level (this applies especially to the shore sites where the pupal cases were located), and hence the advantage of the observed architectural behaviour of adding elements to balance the weight of the case favors its horizontal position on the bottom of the brook. This survival strategy increases the probability that the insect will remain submerged in the water during development and until the adult emerges and flies.

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