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Micro-computerized tomographic observation of the spinning apparatus in *Bombyx mori* silkworms

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ABSTRACT

Silkworms and spiders have evolved complex spinning apparatus thought to use highly controlled conditions to optimize protein folding and crystallization to provide a tough fiber. Accordingly, the structure and function of the natural spinning apparatus has been studied with great attention as an interesting piece of biological engineering with potential for mimicry in an industrial process. However it is still not well understood. Here we used Micro-Computerized Tomographic equipment (mCT) to visualize the three-dimensional structure of the spinning apparatus in *Bombyx mori* silkworms. Multi-directional tomograms obtained by X-ray radioscopy provided valuable information on the detailed arrangement of each muscle of the silk press. It is suggested that the duct in the silk press part plays a part as an extrusion die whose cross-sectional area can be controlled by muscles to optimize applied stresses in the partially gelled silk within its lumen.

1. Introduction

Bombyx mori silkworms produce silk fibroin fiber with outstanding mechanical properties despite its extrusion at room temperature and from an aqueous solution [1,2]. Silk fibroin also possesses impressive biological properties, giving it considerable potential as a biomaterial for tissue engineering [3]. Consequently, great attention has been focused on the natural mechanism of fiber formation with the aim of understanding how tough fibers are produced naturally and how the process might be mimicked industrially.

An important approach is the study of the functional anatomy of the spinning apparatus and there are several reported studies on this [4–6]. Light microscopy of transverse sections has furnished a common description of the spinning apparatus in the *B. mori* silkworm as follows. The spinneret is divided into three consecutive parts: a common duct (150–200 μm and 2000–2500 μm^2 in length and cross-section, respectively); the muscular silk press (300–350 μm and 2000–500 μm^2) and the exit spigot (350–400 μm and 500–800 μm^2) [4,6]. The common duct receives silk proteins from the two separate silk ducts and has a rather wide lumen which is abruptly flattened dorso-ventrally giving it an ellipsoidal cross-section. The common duct is continuous with the duct of the silk press, a complex structure on to which several distinct muscles insert. The spinning tube runs from the silk press duct to the exit spigot and has a somewhat wider lumen with an approximately circular cross-section. The arrangement of the muscles and associated structures in the silk press proper has been investigated. The cuticle lining of the silk press lumen is reinforced by two stiff plates, a short dorsal one and a longer ventral one. The dorsal and ventral muscles have their insertions on the cuticle lining close to the stiff plates and their origins on the part of the external cuticle that surrounds the silk press. These morphological observations suggest that muscles and the associated the stiff plates play a role in adjusting the lumen of the silk press to regulate shear and/or extensional flow in the silk during spinning [6,7]. This hypothesis has been supported by birefringence measurements on the nascent silk threads in situ [8,9].

Although the conventional microscopical work using fixed, embedded and sectioned material studies have provided some insight, the extent to which this deforms the delicate structure of the spinning apparatus is not known. In addition the use of transverse sections means that structures in other planes may be overlooked, for example the detailed arrangement of the muscles. Accordingly we have employed a nondestructive analytical technique utilizing X-ray radioscopy. Recent advances in X-ray microscopy give a spatial resolution of 5 μm, sufficient to resolve many features of the spinning apparatus of silkworms.

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Computerized X-ray micro-tomography is proving increasingly useful in biological and medical research [10,11]. In the present paper, multidirectional cross-sectional images of the spinning apparatus were obtained by X-ray micro-radiography from the same specimen and these were used to create tomograms and computer assisted three-dimensional reconstructions. The functional anatomy of the spinning apparatus with its associated muscles is discussed in relation to previous results obtained by microtomy.

2. Material and methods

2.1. Sample preparation

Domestic *B. mori* larval silkworms were reared on an artificial diet supplemented with fresh mulberry leaves. Two 7-days-old 5th instar *B. mori* larvae that had just started to spin were frozen at −20°C for a day in a deep freezer. To enhance contrast, frozen specimens were freeze-dried by transferring them to liquid nitrogen and then with some liquid nitrogen to the flask of a rotary evaporator which was not warmed in a water bath. These conditions though not optimized, proved satisfactory for determining the arrangement of muscles and cuticlular structures of the spinning apparatus. The cuticle of the body of the silkworms and head capsule but not the labrum was removed with a scalpel from the freeze-dried material before observation in order to avoid its absorption of X-rays.

2.2. Micro-CT observation

Micro-CT experiments were performed on an SMX-100CT Micro-focus X-ray CT system (Shimadzu Co. Inc., Japan). The observations were made at room temperature, typically for several minutes with a focal spot size of 5 μm and an X-ray tube voltage of 35 kV. Fig. 1 shows the schematic illustration of Micro-focus X-ray CT system. The samples were rotated on the stage and the radiolucence obtained for each direction. Tomograms for each direction were obtained with a long vertical motion of the sample stage. Three-dimensional reconstructions were prepared from the tomogram slices using the software TRI-3D/BON (RATOC System Engineering Co. Inc., Japan) [6,7,12].

3. Results and discussion

3.1. The arrangement of the muscles of the silk press

Tomograms of the silk press and its muscles are shown in Fig. 2(A1–4, B1–4, C1–3) and 3 (t1–11) and three-dimensional

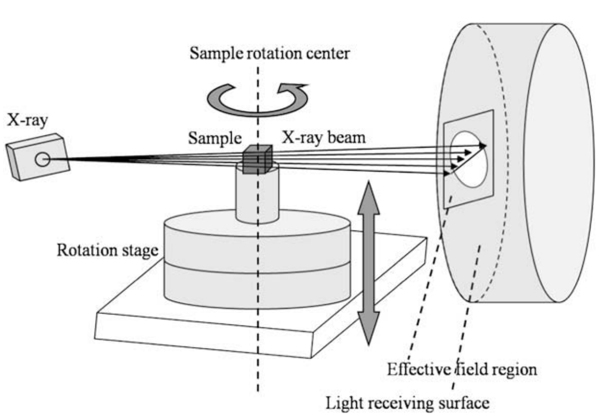

Fig. 1. Schematic illustration of mCT system.

reconstructions from tomogram slices in Fig. 2(a,b). Two main sets of muscles, the dorsal and ventral muscles are associated with the silk press and have their insertions on the cuticle lining of the duct of the silk press. Whole mounts show that each muscle fascicle of the silk press has a coherent sarcomeric banding pattern and therefore represents an individual muscle cell. The left and right dorsal muscles each consist of a longitudinal row of five to six more-or-less separate fascicles (Fig. 2B1–4). In contrast, the left and right ventral muscle fascicles are much smaller than the dorsal ones and each consists of only one or two fascicles in a longitudinal row. The dorsal muscles are best seen in Fig. 2b, A1 and B1 and insert via short tendon-like extensions into the dorsal midline of the duct of the silk press (Fig. 2A1–4, B1; 3t7, h7, h4). The ventral muscles insert via short tendon-like extension attached to the dorso-lateral margins of the cuticle lining of the silk press's duct as seen in Fig. 2a and A2–4; 3 t8 and h9. Both sets of muscles have their origins on a thickened ring of cuticle best seen in Fig. 2B1–4 and C1–3. The plane of the ring of cuticle tilts forward and downwards at an angle of about 55° to the horizontal axis. It is formed as a thickening of the cuticle of the labrum, a structure modified from the silkworm's lower lip which contains the silk press and carries the base of the exit spigot. The origins of the dorsal muscles are found dorso-laterally on the thickened ring of cuticle and are best seen in Fig. 2a and b while those of the ventral muscles are found ventro-laterally also on the thickened cuticle ring and are best seen in Fig. 2A3 and C2. The fascicles of the dorsal muscles therefore run more or less dorso-ventrally but with a marked tilt inwards and slightly forward from origin to insertion (Fig. 2B1–2). The ventral muscles also tilt, inwards and forwards from origin to insertion but are arranged more obliquely than the dorsal muscles when viewed in the transverse plane (Fig. 2A1–3). In addition to the dorsal and ventral muscles, one to two small lateral muscles on each side, not described in previous studies, run laterally and somewhat anteriorly from their origins on a slight thinning of the cuticular ring just dorso-lateral to the origin of the ventral muscles (see Fig. 2B4 and C2, 3). The lateral muscles insert via a short tendon-like structure on the dorso-lateral margin of the cuticle lining of the lumen of the duct of the silk press, just dorsal to the insertion of the ventral muscles (Fig. 3h9, t8 and 2A2).

From the orientation of the muscles and the position of their origins and insertions it is likely that contraction of both dorsal, ventral and lateral muscles will have three effects, 1–3:1. The radial pull exerted on the cuticle lining of lumen of the silk press duct as we have previously suggested [6], is likely to dilate the lumen of the silk press reducing the shear and extensional forces on the nascent silk brins within the silk press. In this way the muscles of the silk press are thought to regulate the order parameter and crystallinity of the silk. 2. The anterior to posterior tilting of these muscles as seen from vertical longitudinal plane would tend to pull the cuticle lining of the silk press backwards. Such backward movement is likely to result in an antero-posterior stretching of the thin cuticle lining of the spinning tube just anterior to the stiff plates in the silk press, leading to a constriction of the lumen here. Together with the dilation of the lumen of the silk press proper effect 1 mentioned above, this may enable the silk press and spinning duct to act as a micropump to initiate and regulate the flow of liquid or partially gelled silk proteins at the start of spinning and at other times during the spinning process. Thus contraction of the dorsal, ventral and lateral muscles may provide the filling stroke for the micropump (see below). 3. In addition the greater size of the dorsal muscles compared with the ventral ones may serve to pull the duct of the silk press upwards and this may help to constrict the lumen anterior to the stiff plates.

It is likely that the movements described above are reversed on relaxation of the muscles largely by energy stored elastically in the cuticle of the duct and in the ring of thickened cuticle on which the

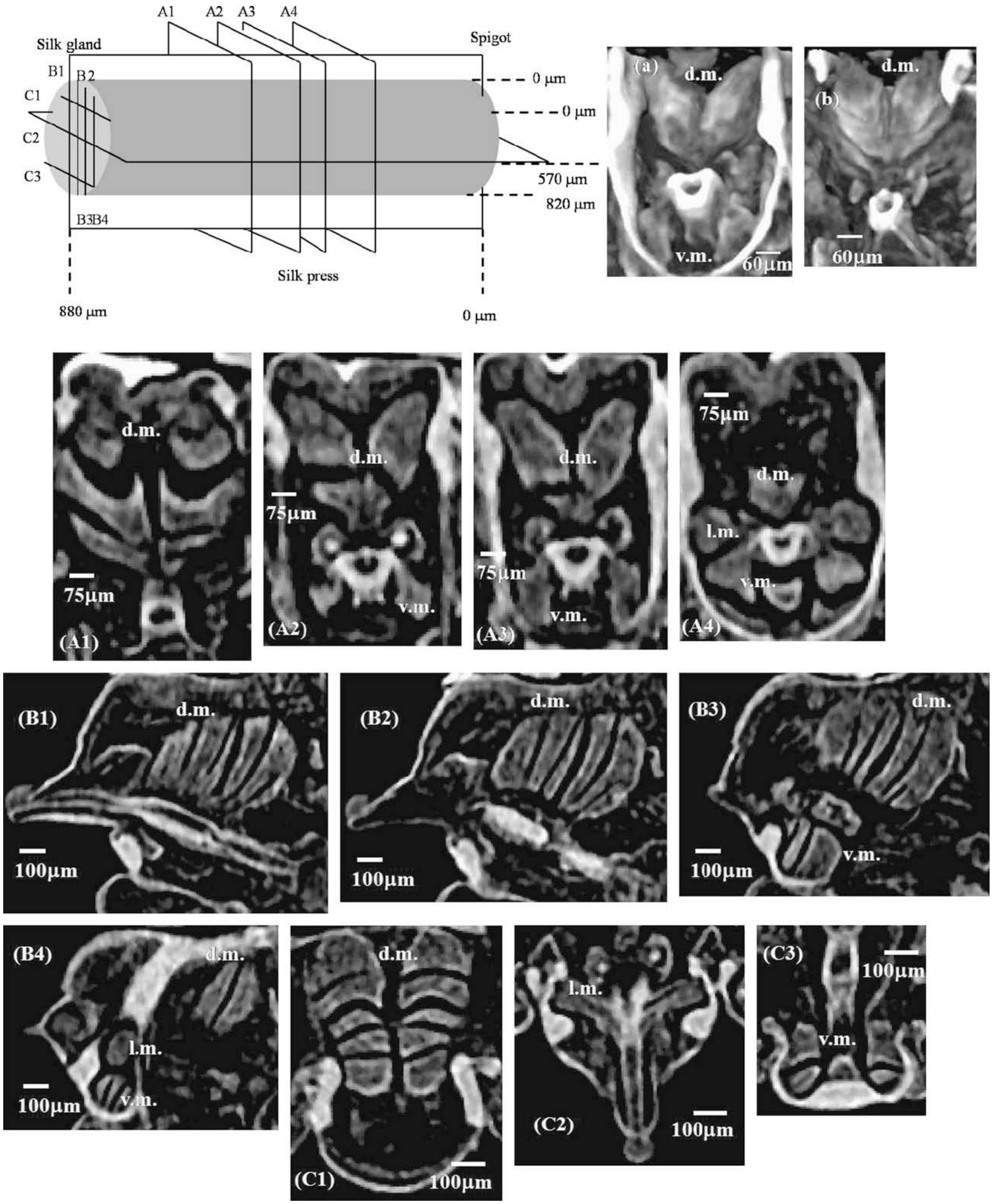

Fig. 2. Tomograms and three-dimensional reconstructions from tomographic slices of the silk press and related structures taken in different planes (a), (b) three-dimensional reconstructions; (A1)–(A4), transverse tomographic slices, (A1) 720 μm, (A2) 540 μm, (A3) 500 μm, (A4) 400 μm from the spigot; (B1)–(B4), vertical longitudinal tomographic slices at the following distances from the datum plane (B1) 305 μm, (B2) 340 μm, (B3) 410 μm, (B4) 480 μm. (C1)–(C3); horizontal longitudinal tomographic slices at the following distance from the horizontal datum plane (C1) 260 μm, (C2) 480 μm, (C3) 680 μm. Abbreviated symbols show d.m. = dorsal muscles, v.m. = ventral muscles and l.m. = lateral muscles.

muscles have their origins. Thus elastic recoil of the cuticle lining the silk press would reduce the gap between the dorsal and ventral stiff plates increasing shear and extensional forces on the nascent silk thread as we have already suggested [6]. An advantage of such an arrangement would be that the dorsal, ventral and lateral muscles would normally be relaxed for most of the time, with a consequent saving in energy compared with an arrangement that required the press to be actively constricted during spinning. Elastic recoil would also provide the emptying stroke to enable the silk press and spinning tube to act as a micropump (see above). Very small muscles (seen clearly in Fig. 3h10 and more faintly in Fig. 2B4 and C2) appear to originate on the membrane of the lateral muscles

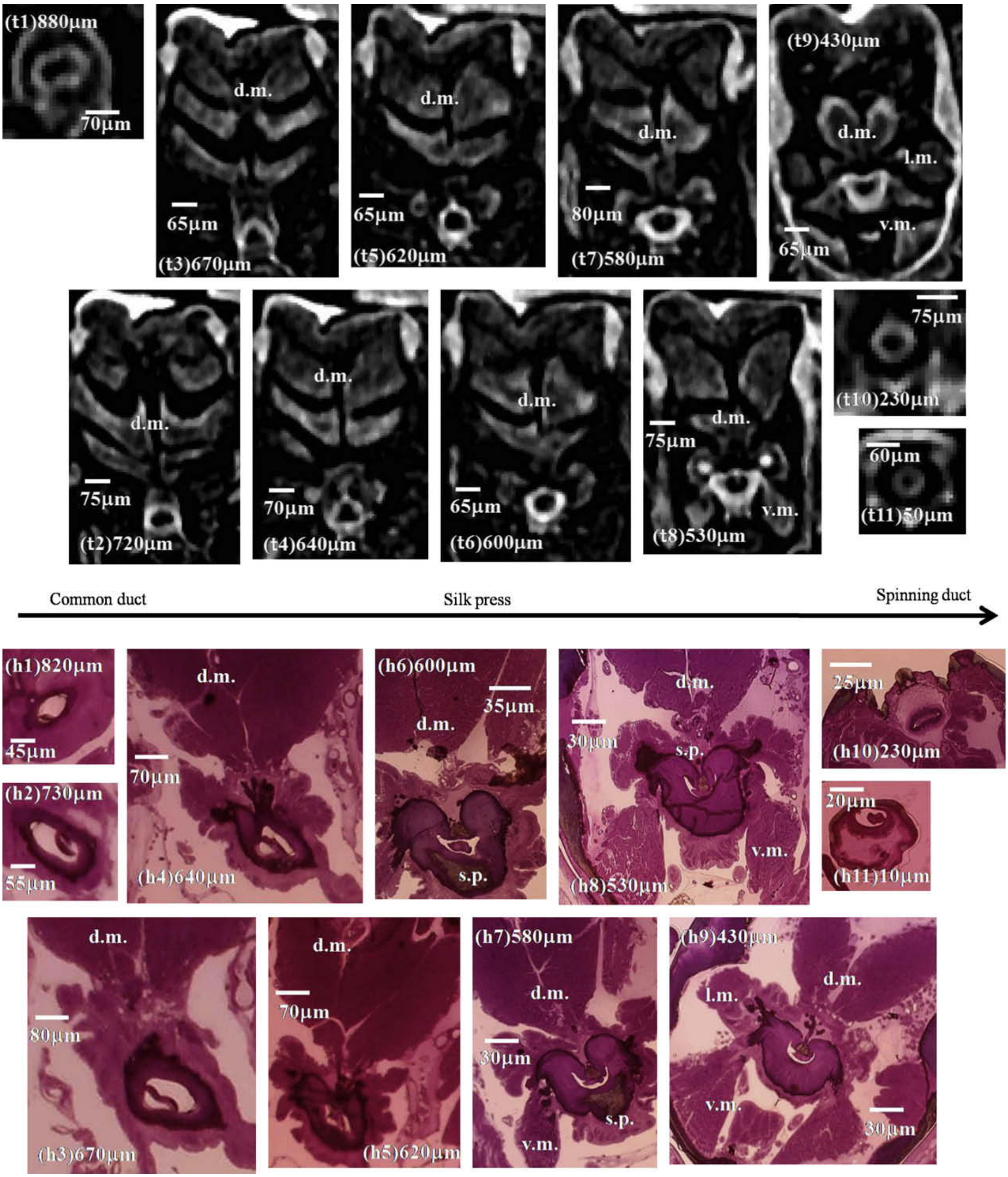

Fig. 3. A comparison between mCT transverse tomographic slices (t) and micrographs of transverse histological sections (h) through the silk press and related structures taken at different distances from the spigot tip. Abbreviated symbols show d.m. = dorsal muscles, v.m. = ventral muscles l.m. = lateral muscles and s.p. = stiff plates.

at a point fairly close to their insertion (see Fig. 2C2) and to run forward to insert on the cuticle close to the base of the spigot. It is possible that these control the movement of the spigot in the horizontal plane and stabilize it relative to the labrum, as an aid to figure-of-eight spinning.

3.2. Comparison of histological and micro-CT observations

Fig. 3 enables comparison of the size and shape of the lumen of the duct in our tomograms with our observations on histological sections from our previous study [6]. Fig. 4 plots the change in cross-sectional area of the duct as a function of the distance from the spigot determined from the two different imaging techniques. Fig. 3t1,2 and h1,2 show that in both histologically sectioned material and in tomograms the lumen of the common duct that lies posterior to the silk press proper is ellipsoidal in cross-section. Here, and in the last 350 µm of the spinning duct the cross-sectional area of the lumen is roughly similar with both methods (Fig. 4). However, in the region of the duct of the silk press (670–370 µm from the spigot), the two different imaging techniques gave markedly different results. In histological sections the lumen of the silk press proper was dorso-ventrally flattened and bow-shaped

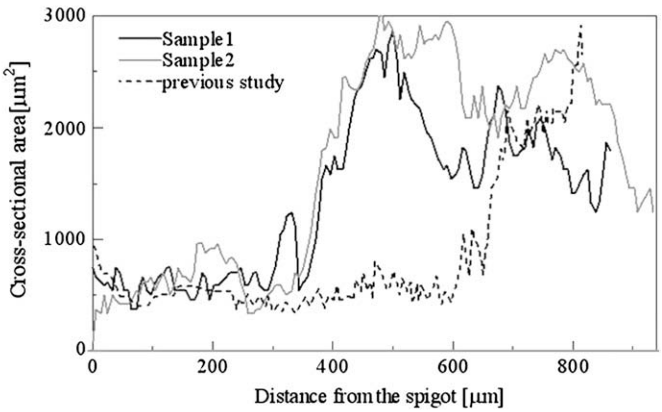

Fig. 4. Changes in the cross-sectional area (μm^2) of the lumen in the spinneret as a function of the distance from the spigot. Solid lines show results from mCT observations from two different specimens and dotted line, from histological sections.

compared with the roughly circular cross-section of the lumen in tomograms (compare Fig. 3t7 and c7) and the cross-sectional area lumen was markedly less than that in tomograms of this region (Fig. 4). In histological sections the cross-sectional area dropped markedly at about 670 μm close to the start of the silk press and remained at a fairly constant level until it dilated in the spigot. In contrast, in both tomographic specimens the cross-sectional area of the lumen in the silk press *per se* was markedly higher compared with the histological material, increasing markedly from a position about 610 μm and peaking at position about 500 μm in the middle of the silk press before returning at a distance of about 360 μm to a value close to that seen in histological sections (Fig. 4). These morphological differences can be readily explained by differences in the two preparation techniques. We suggest that the glutaraldehyde fixation and epoxy resin embedding used for histological preparation maintains the lumen of the silk press in the recoiled state by relaxing the muscles of the silk press or preventing them from contracting. We also suggest that the slow freezing for the tomographic observations causes the muscles of the silk press to contract, dilating the lumen of the silk press and changing its shape to approximately circular in cross-section. In this connection there is an ample evidence that glutaraldehyde fixation can be used to preserve muscles in a contracted or relaxed state but can under certain circumstances relax naturally contracted or tetanized muscle fibers [13]. In addition there is good evidence that slow freezing initiates muscle contraction [14] presumably by the release Ca^{2+} ions to the myosin from ice-damaged sarcoplasmic reticulum.

4. Conclusion

Using tomographic sections taken in different planes and three-dimensional reconstructions based on these we determined in some detail, the position of the origins and insertions and the course of the dorsal, ventral and newly discovered lateral muscles. We also determined the relation of these muscles to the cuticle of the lumen and of a newly discovered cuticular thickening in the exoskeleton of the labrum surrounding the silk press. From the position and direction of muscles, we discussed the effects of each muscle. We suggest that these muscles, opposed by elastic recoil in the cuticle of the duct of the silk press and thickened ring in the cuticle of the labrum, are thought to control the cross-sectional area and cross-sectional shape of the silk press. This is likely to enable the silkworm to modulate the order parameter and crystallinity of the silk as it is formed by regulating shear and/or extensional flow in the silk. We also suggest that the muscles and cuticular structures associated with the silk press may enable it to be used as a micropump to initiate spinning and to control the flow of liquid or partially gelled silk in the spinning process.

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