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Visualizing the postembryonic development of *Sarcophaga peregrina* (flesh fly) by NMR microscopy

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Abstract. The postembryonic development of the flesh fly was studied using high resolution nuclear magnetic resonance imaging. Because this development occurs in a puparium, this process cannot be observed directly using standard histological techniques. The remodelling of histolysing larval tissues to developing imaginal tissues including the yellow body, a transient alimentary structure, and the integration of the developed adult structure were revealed in the images. Most surprisingly it was found that a large gas space that forms in the central region of the prepupa moves to the dorso-anterial region in less than 5 min due to the larval–pupal apolysis together with separation of the developing pupal epidermis from the puparium.

Key words. Flesh fly, NMR microscopy, postembryonic development, *Sarcophaga peregrina*.

Introduction

The understanding of insect postembryonic development, especially when the pupal stage develops in a puparium, is hampered by the invasive nature of standard histological techniques. As yet, very few applications of NMR microscopy to the important field of insect development have appeared (e.g. Gassner & Lohman, 1987; Conner *et al*., 1988; Goodman *et al*., 1995; Mapelli *et al*., 1997) because only with the advent of high resolution nuclear magnetic resonance (NMR) imaging (NMR microscopy) (Callaghan, 1991; Price, 1998) has it become possible to acquire images with sufficient resolution. Here we present the first detailed study of the postembryonic development of a dipteran insect, *Sarcophaga peregrina* (flesh fly), using NMR microscopy. This non-invasive technique allowed the pupal development to be studied continuously in the one pupa. Although pupal development of imaginal discs/glands has been characterized in *Drosophila* (Bate & Arias, 1993), there is little knowledge of the entire process of postembryonic development including the remodelling of histolysing larval tissues to developing imaginal tissues and the integration of the developed adult structure. The flesh fly is suitable for investigating the sequential and harmonious process of metamorphosis because ecdysis can be initiated (and thus synchronized) simply by drying the larva. Moreover, because it is about 100-fold greater in volume than *Drosophila melanogaster*, the rapid recording of images with organ level detail is possible, which is necessary for observing the process of metamorphosis. Observation of serial images revealed the dynamic movement and integration of the developing imaginal tissues as well as histolysis of larval tissues. Interestingly, the large gas space that forms in the central region of the prepupa moves to the dorso-anterial region in less than 5 min due to the larval–pupal apolysis together with separation of the developing pupal epidermis from the puparium.

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Materials and Methods

Flesh flies were reared according to the method of Ohtaki (1966). Third-instar larvae were kept under wet conditions at ambient temperature (~ 25°C) to prevent pupariation. Approximately 12 h after drying they started pupariation synchronously. ^{1}H-NMR microscopy was conducted on a Bruker DRX 300 NMR spectrometer operating at 300 MHz using a 5-mm insert (Callaghan, 1991; Price, 1998). For conducting imaging experiments, pupae (one per tube) were placed in 5-mm (o.d.) NMR tubes. After being placed in the imaging probe, the NMR tube was not moved for the duration of each series of images. Thus, any motion observed in the

images results from the motion of the pupa itself. All of the images were acquired using a multispin multiecho imaging sequence at ambient temperature (~ 21°C). Three images were acquired simultaneously with the distance between successive imaging planes being 0.5 mm (only the central image is shown in the figures). For the longitudinal images a field of view of 12 mm × 12 mm was used. The field of view was digitized into 256 pixels in each direction, giving an in-plane resolution of 47 µm. For the transverse images a field of view of 5 mm × 5 mm was digitized into 64 pixels in each direction, giving an in-plane resolution of 78 µm. A slice thickness of 0.5 mm was used in all cases. Typical image acquisition parameters were a recycle delay of 800 ms and an echo time of 9.9 ms in the case of 256 × 256 pixel images.

More than 10 pupae were used in preliminary experiments to determine a timetable for acquiring the images and suitable experimental parameters that would clearly document their development. In each case, allowing for some slight natural variation in the timing of the development of the individual pupae, the NMR images of the pupal development were quite reproducible.

Results and Discussion

Different transverse and longitudinal images of the same pupal stage are shown in Fig. 1. Many of the developing adult

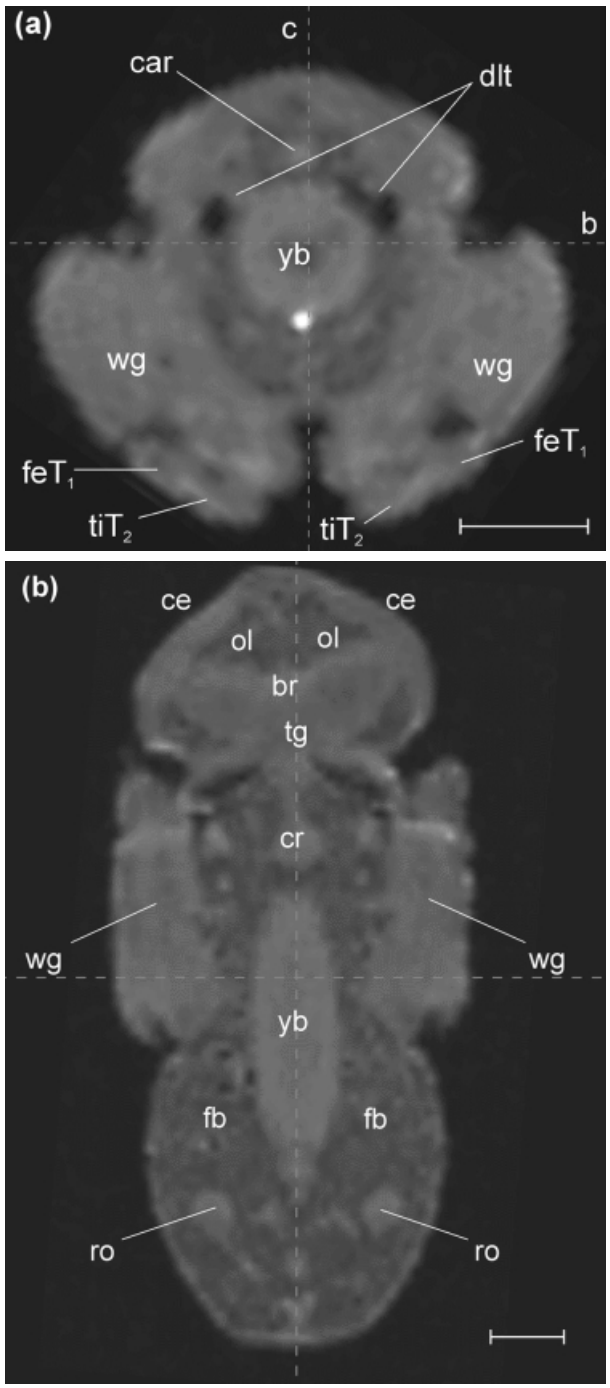
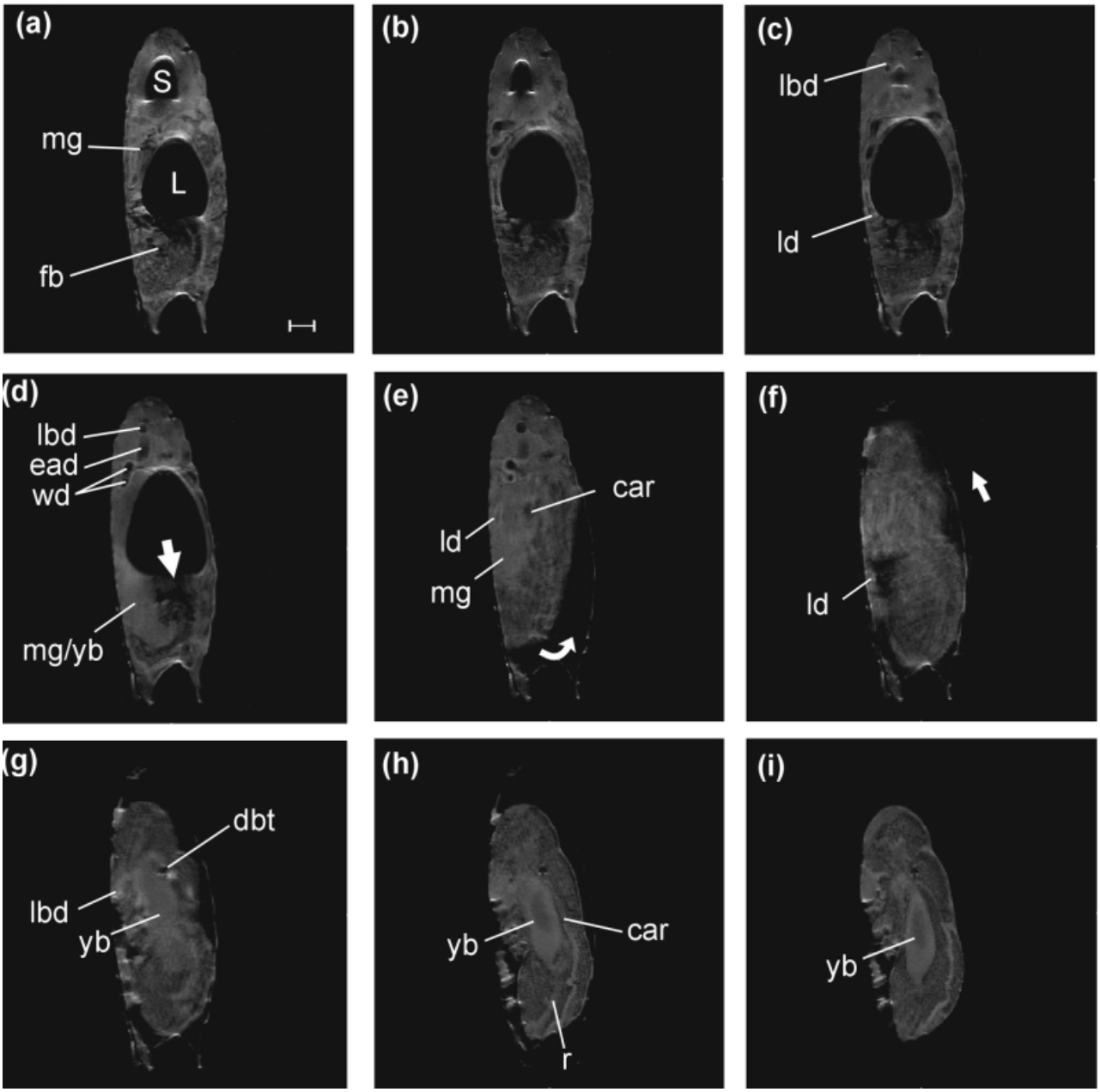


Fig. 1. Transverse (a) and two different longitudinal images ((b) posterior view and (c) lateral view) of the same pupa at a late stage in pupal development. In (a) the top of the figure corresponds to the dorsal side and the dashed lines labelled 'b' and 'c' denote the orientations of (b) and (c). In (b) and (c) the mouth aperture is towards the top of the figure. The bright spot near the middle in (a) and the vertical stripe near the middle of (c) are artefacts. The white bars denote 1 mm. Various parts of the anatomy have been identified and these are denoted by: antenna (ant); brain (br); cardia (car); compound eye (ce); coxa (cx); crop (cr); dorsal air sac of the thorax (das); dorsal lateral tracheae (dlt); oesophagus (es); fat body (fb); femur (fe); head air sac (has); hindgut/rectum (hg/r); labellum (lab); Malphigian tubules (mt); optic lobe (ol); pharynx/cibarium (ph); reproductive organ (ro); salivary gland (sg); tarsus (tar); thoracic ganglion (tg); tibia (ti); T_1, T_2, T_3 first, second and third thoracic segments, respectively; wing (wg) and yellow body (yb).

structures can be identified from their shape and location in the pupa. At the pupal stage, many larval tissues are histolysing, whereas imaginal discs are developing. In the case of digestive organs, however, both events occur together in one transient sac called the 'yellow body' (yb in Fig. 1). It was reported (Skaer, 1993) that in the metamorphosis of the *Drosophila* gut the degeneration of the larval gut is accompanied by the outgrowth of the new epithelium, and only in the midgut does regeneration precede loss, so that the epithelium of the larval midgut is sloughed off into the lumen of the new midgut, to form the yellow body as shown in Fig. 1. In this structure, midgut remodelling occurs and it is finally evacuated as the meconium after eclosion of the adult.

A series of images showing the development of the fly through various stages of pupal development is presented in Fig. 2. The changes that occur at each stage are described in detail in the figure caption. During the early stages of pupation the whole pupa including the pupal case is visible. However, as development progresses the pupal case becomes invisible. This probably reflects loss of water from the pupal tissue and the shortening of the spin-spin relaxation time with the increasing dehydration. Initially two gas holes ('bubbles') formed; a small gas hole near the mouth aperture and a larger gas hole in the midgut region. The smaller hole closed earlier than the large gas hole. The large gas hole disappeared in less than 30 min (see Fig. 2). This was the period of most drastic change in



pupal development. The 'arrowhead'-shaped brightness patterns around the gas holes are artefacts arising from susceptibility differences between the gas and surrounding tissues (Callaghan, 1990, 1996). A 'video', in the gif computer graphics format, which is in effect a greatly expanded version of Fig. 2, is available from the authors on request.

To investigate further this period of very rapid structural change, another pupa was more rapidly imaged (i.e. acquired at lower resolution) in order to reduce motional blurring during the image acquisition and the images were acquired at intervals as short as 5 min (data not shown). It was found that the disappearance of the large gas hole is associated with movement of the pupa inside the puparium. Astonishingly, this change occurs in less than 5 min. Previously, it has been reported that in *Drosophila* the bubble is gradually displaced to the anterior portion of the puparium and the duration of the gas movement was estimated to be less than 12 min during the larval/apolysis (Bainbridge & Bownes, 1981). In the present study, after the posterior gas chamber (i.e. located between the pupal cuticle and the larval endo cuticle) was expelled (Fig. 2c), head eversion occurred and the central bubble suddenly leaked to the posterior (Fig. 2d), then moved to the dorsal side (Fig. 2e) and finally to the anterior (Fig. 2f). This side became the dorsal part of the adult. Prior to the disappearance of the large hole, it was difficult to correlate the pupal outline and adult anatomy. However, immediately after disappearance of the large gas hole it was possible to discern the outline (i.e. three parts of the adult structure: head, thorax and abdomen) of the nearly complete fly. It appears that physical separation of successive cuticles occurred at this stage. Disappearance of the large hole preceded full extension of the wings and legs along the abdomen in the phanerocephalic pupa (Fig. 2g). It seems that the gas movement controls the spatial and temporal development of the imaginal discs.

It appears that the major processes of metamorphosis in *Sarcophaga peregrina* are similar to those in *Drosophila*, at least during the period observed using NMR microscopy (within 2.5 days after drying). However, the duration was more than twice that of *Drosophila*: 216 h at 27°C (unpublished observation); or 103 h at 25°C (Bainbridge & Bownes, 1981). Imaging started about 24 h after the drying of the larva. Normally 12 h after drying, *Sarcophaga* larvae enter the so-called white pupal stage that is, in the case of *Drosophila*, denoted as 0 h WP. Taking this as the baseline, the movement of the central bubble is assumed to occur after about 45 h. The bubble in *Sarcophaga* pupa disappears later than estimated from the timing in *Drosophila* pupa (Bainbridge & Bownes, 1981) (stage P4(ii) 12–13.5 h). We assume that the delay of the gas hole disappearance is related to, amongst other factors, the difference in species and the lower temperature (21°C) used in the present study. Nevertheless, even after performing the imaging it remains unclear how the bubble forms in the pupa. To clarify this we dissected a pupa to see the bubble. The large bubble was not enclosed within any tissue, whereas the small gas hole was within the dorsal pouch (discussed below). It seems that the central bubble transiently has a function similar to that of the 'air bladder' in fish because it is reported that in *Drosophila* the bubble appears to be associated with the trachea when dissected out and that it failed to develop in submerged prepupae (Bainbridge & Bownes, 1981).

The serial NMR images allow the poorly understood process of integration of the many separate parts into one complete fly body during metamorphosis to be visualized dynamically. The eversion of the imaginal head sac, as well as those of the imaginal legs and wings (which exist near the imaginal head sac at this period), occur simultaneously in the anterior part of the 'prepupa' before the disappearance of the large bubble. Thus, it seems that the disappearance of the first small bubble in the anterior portion makes sufficient space for the eversion of the imaginal discs. In the head region, the integration of the independent discs occurs around a dorsal pouch, which appears as a small gas hole in the NMR images. As distinct from the large gas hole, the dorsal pouch is surrounded by water. This means that this air space is surrounded by some discs. In the region surrounding the dorsal pouch, many imaginal head discs are starting to develop. As shown in Fig. 2c, a labial disc is located anterior-ventral outside of the dorsal pouch and eye-antennal discs are located at the posterior-ventral side of the

Fig. 2. A series of longitudinal images detailing the stage of pupal development where the gas spaces disappear. The white bar denotes 1 mm. The arrows denote the direction of gas flow. (a) 12 h after puparium formation (i.e. 12 h AP), the small gas hole (S) in the head region and the large hole (L) in the central region were created before puparium formation. The abdominal region contains the (as yet) undissociated fat body (fb). The larval midgut (mg) region is on the larval side of the large hole. Many discs have started development and larval muscles, especially in the dorsal part, are degrading. (b) 29 h AP, the small gas hole starts shrinking and the development of discs around this hole proceeds. (c) 40 h AP, the small gas hole disappears and discs composed of the head structure (e.g. the labial disc; lbd) exist in the head region and the leg discs (ld) are expanding and are visible as pouches. (d) 45 h AP, head eversion is completed and the ventral midgut (mg) moves to the frontal posterior. Two small lengthened holes in the ventro-anterial region are wing disc (wd) pouches. The elongated leg discs move further toward the ventral medial region. The brain and ganglia are visible in the central head zone and the eye-antennal discs (ead) and labial discs (lbd) become larger. (e) 46 h AP, the blurring indicated the motion of the gas from the large gas hole. The head structure looks similar as in (d) but the developing structures in the thorax and abdomen have been pushed towards the centre. The cardia (car) becomes visible at the dorsal side of the midgut (mg). (f) 47 h AP, larval/pupal apolysis is complete. Three parts of the adult structure (head, thorax and abdomen) are now distinguishable in the correct position. Leg discs (ld) start eversion. (g) 48 h AP, the yellow body (yb) moves to the central region. Each part of the leg discs are distinguishable. The alimentary canal is not complete. The dorsal branch tracheae (dbt) is visible. The fully developed labial disc (lbd) can be identified as the ventral tips of the head. (h) 73 h AP, the gas ventilation has ceased and the yellow body (yb) contains the digested larval midgut. The adult midgut tissues on the outer side of the yellow body are completely delineated. The rectum (r) reaches to the posterior terminal. The tibia dries and hardens. (i) 101 h AP, many organs are now integrated together in the correct positions of the adult structure. The yellow body (yb) moves down to the abdomen. Pupal apolysis is complete.

dorsal pouch. At this stage, the labial and eye-antennal discs are separated. With the gas diminishing in the dorsal pouch, both of these discs are enlarging and becoming closer to each other (see Fig. 2d) and finally integrate into a contiguous cell layer, the head sac (see Fig. 2e).

Using NMR microscopy we have directly visualized the ordinarily invisible process of pupal metamorphosis. This allows a much more detailed understanding of the process of the remodelling of histolysing larval tissues to developing imaginal tissues and the integration of the developed adult structure. In particular, gas flow inside the pupa was found to be an important element in the spatial control of metamorphosis.

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