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Functional morphology of the heart and of a new cephalic pulsatile organ in the blowfly *Calliphora vicina* (Diptera: Calliphoridae) and their roles in hemolymph transport and tracheal ventilation

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Abstract

In the blowfly *Calliphora vicina* (Diptera: Calliphoridae), the morphology of the dorsal vessel and of a new cephalic accessory pulsatile organ (CPO) were analysed with light-microscopic, SEM and TEM techniques. The CPO and neck aorta are reconstructed 3-dimensionally by computer-aided design. The pulse activity of the CPO and of the heart was measured in intact flies over periods of several hours or days using contact-thermography with laser beam heat-marking. The intratracheal pressure was simultaneously measured at the anterior thoracic spiracle. The dorsal vessel is constructed of pairs of left–right alternating cells. Its enlarged chamber in the anterior abdomen contains two pairs of incurrent ostia, its posterior narrower heart tube possesses three pairs of incurrent ostia and paired caudal excurrent openings. The aorta opens with a funnel-like opening in the neck. Proportions, arrangement and ultrastructure of the aorta, heart cells and pericardial muscles are described. Cushionlike sarcoplasmic protrusions of heart cells (pair no. 17) probably function as internal valves. The neck aorta is constructed of a cuticular 'roof' deviating from the dorsal neck membrane and a ventral longitudinal muscle 'floor'. The aorta is not kept open because of missing muscle or connective tissue strands. The underside of the CPO is fused with air sacs that function as antagonists to the muscles. The heart reverses its beat periodically in resting and active flies. During the longer forward-pulse periods, mean frequency is lower (about 3.0 Hz at Ta 20°C), during the shorter backward periods mean frequency is higher (4.6 Hz). The CPO beats only during forward-pulse periods of the heart with an independent and slower pulse rate (1.8 Hz). The CPO-pulses produce positive pressure pulses at the anterior thoracic spiracle. During backward-pulse periods of the heart and pulse pause of the CPO, a continuous negative pressure arises at the thoracic spiracle instead of pressure pulses. The intimate connection of an accessory pulsatile organ with tracheal air sacs makes it work as a bifunctional pump for hemolymph distribution and tracheal ventilation. Neurosecretory and synapsing innervation of the CPO in connection with aorta, heart and pericardial septum muscle innervation suggest that both organs are regulated and that the duration of their periods is neuronally coordinated.

Keywords: Heartbeat reversal; Circulation; Respiration; Pulsatile organ; Heart cells

1. Introduction

Although higher flies are one of the most successful and powerful flying insect groups, the organs of their circulatory system are only incompletely described and the function of the entire circulatory system is not understood. Morphological studies concentrated upon the larval structures (Pantel, 1914; Ludwig, 1949; Ranade, 1967; Jensen, 1973) and aspects of metamorphosis (Weismann, 1864; Kowalewsky, 1886; Vaney, 1902; Whitten, 1962) or innervation of heart and pericardial septum muscles with respect to the regulation of pulse rate (Normann, 1972; Meola and Cook, 1986). Older light microscopic studies dealing also with heart morphology of the adult, give an imprecise impression (Lowne, 1893–95, Hewitt, 1910; Miller, 1950) or refer to special details as anterior heart chamber (Jensen, 1973) and the 'wing hearts' (Thomsen, 1938; Krenn and Pass, 1995). Ultrastructural research of the myocardium wall was done in *Stomoxys calcitrans* (Cook and Meola, 1983). Consequently the dorsal vessel was never analysed along its total length with its different parts. The number and position of incurrent ostia and excurrent openings is described controversially.

Another group of papers deals with regulation of the fly heart carried out on semi-isolated heart preparations (Normann and Duve, 1969; Normann, 1972; Brazeau and Campan, 1970; Thon 1980; Cook and Meola, 1983; Angioy and Pietra, 1995). Normann (1972) stated that

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"activity of the heart appeared very different in intact flies and in semi-isolated preparations". Thus even the fundamental question, whether there are only frequency fluctuations in heart rhythm or if there are heartbeat reversals, is controversial. Reversals were not seen by Normann (1972), Cook and Meola (1983) and Duve et al. (1993). While in isolated heart preparations, backward-pulse direction was attributed to the slow phase by several authors (Brazeau and Campan, 1970; Queinnec and Campan, 1975; Thon and Queinnec, 1976; Thon, 1980, 1982; Angioy and Pietra 1995), in intact flies, using contact thermography with heat marking techniques, the 'fast phases' were attributed to be backward pulses (Wasserthal, 1982b; 1996).

To understand the function of the dorsal vessel in the intact fly and its adaptations to alternating pulse activity, a more detailed morphological analysis was performed along the entire dorsal vessel.

2. Material and methods

The tissues were fixed by injection of 4% glutaraldehyde/paraformaldehyde into the insect, buffered in 0.08 M phosphate (pH 7.2) with 4% sucrose. After dissection at room temperature subsequent storage in the fixative at 4° C for 2 h and postfixation with 1% $0s0_4$ in phosphate buffer at 4°C for 1.5 h, specimens were embedded in Epon. Ultrathin sections were cut with glass and diamond knives on a Reichert Ultracut microtome, stained in 1% uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined in a Zeiss EM 10 TEM. The semi-thin sections were stained with toluidine blue and photographed with a Zeiss Axiophot.

Whole mount preparations of the heart and aorta for identification of cell arrangement were stained with orceine acid and toluidine blue.

For SEM analysis the samples were dissected and washed in saline solution (Kaissling and Thorson, 1980). They were fixed as mentioned above, but left in glutaraldehyde at 4°C for 1–2 weeks with repeated changes of the fixative. After dehydration and impregnation with organic material with a low melting point of 40–45°C (paraffine or camphene), the specimens were cut. After removal of the embedding material, the specimens were gold-sputtered and examined in an ISI IIIa and Hitachi S-800 SEM.

For 3-D computer reconstruction, the scanned drawings of the contours of the relevant structures in the semithin sections were vectorized by Freehand 5.51 and imported, modelled and rendered by Ray Dream Designer 4.1 on a Mac Quadra 950.

The contact thermographs were performed with microthermistors (Veco 32a, 402a, 2kΩ) as described earlier (Wasserthal, 1980a). The flies were elastically glued to a stick at the anterior scutum by Pattex. A light-weight running ball of styrofoam gave them the 'illusion' of free mobility. For establishing a temperature gradient hemolymph was locally heat-marked to an excess temperature of 1.5°C above fly body temperature (= ambient between 20 and 25°C) using a 30 W He–Ne-laser beam. Intratracheal pressure was measured with fine microcapillaries adapted to the spiracular opening of the anterior thoracic spiracle and connected to a Sensym pressure sensor SCXL004DN calibrated with a technical-manometer (1 millibar full scale). All signals were amplified and data were sampled with a rate of 40 events s^{-1} by MacLab-Interface on a Mac computer. Heart activity was recorded in 19 individuals. The CPO activity and pressure measurements at the thoracic spiracle were measured in three individuals during 4 weeks.

3. Morphological results

The dorsal vessel of the blowfly consists of three morphologically different regions (Figs 1a, 3b): (1) the enlarged chamber in the anterior abdomen with two pairs of incurrent ostia; (2) the posterior heart tube with three pairs of incurrent ostia and caudal excurrent openings and (3) the (thoracic) aorta without ostia terminating with one excurrent opening in the neck. As a fourth structure in the occipital head an accessory pulsatile organ is associated with the aorta.

The dorsal vessel is built up of two rows of large hexagonal cells which contact at their dorsal and ventral sides (Fig. 3a). The muscle fibres continue over the cell borders by intercalated discs. Their large, probably polyploid nuclei surrounded by sarcoplasm bulge into the lumen (Figs 2b,c). The anterior heart chamber is constructed of 20 cells of a total of 54 (abdominal) heart cells (Fig. 3b). Among these cells at intervals of four to two pairs of wall cells, two semilunar cells protrude on each side into the heart lumen forming an incurrent ostium. The nuclei of these ostial flaps are smaller than those of the heart wall and have probably a lower degree of polyploidy. The ostia are staggered because they are interspersed between alternating consecutive heart wall cells of both sides.

3.1. Heart chamber

The heart chamber in the anterior abdomen (Figs 1, 2a) has a diameter of about 300–450 μm, a length of 1000–1200 μm and a wall thickness of 8–10 μm when relaxed (Fig. 4a). The wall of the heart chamber consists of a single cell layer with densely arranged spiral muscle fibres. They contain about 50% muscle fibrils more peripherally and 50% 'normal' sarcoplasm with mitochondria more centrally. The lumen face is coated with a simple basement membrane. The hemocoel face is lined by a basement-membrane-like connective tissue layer

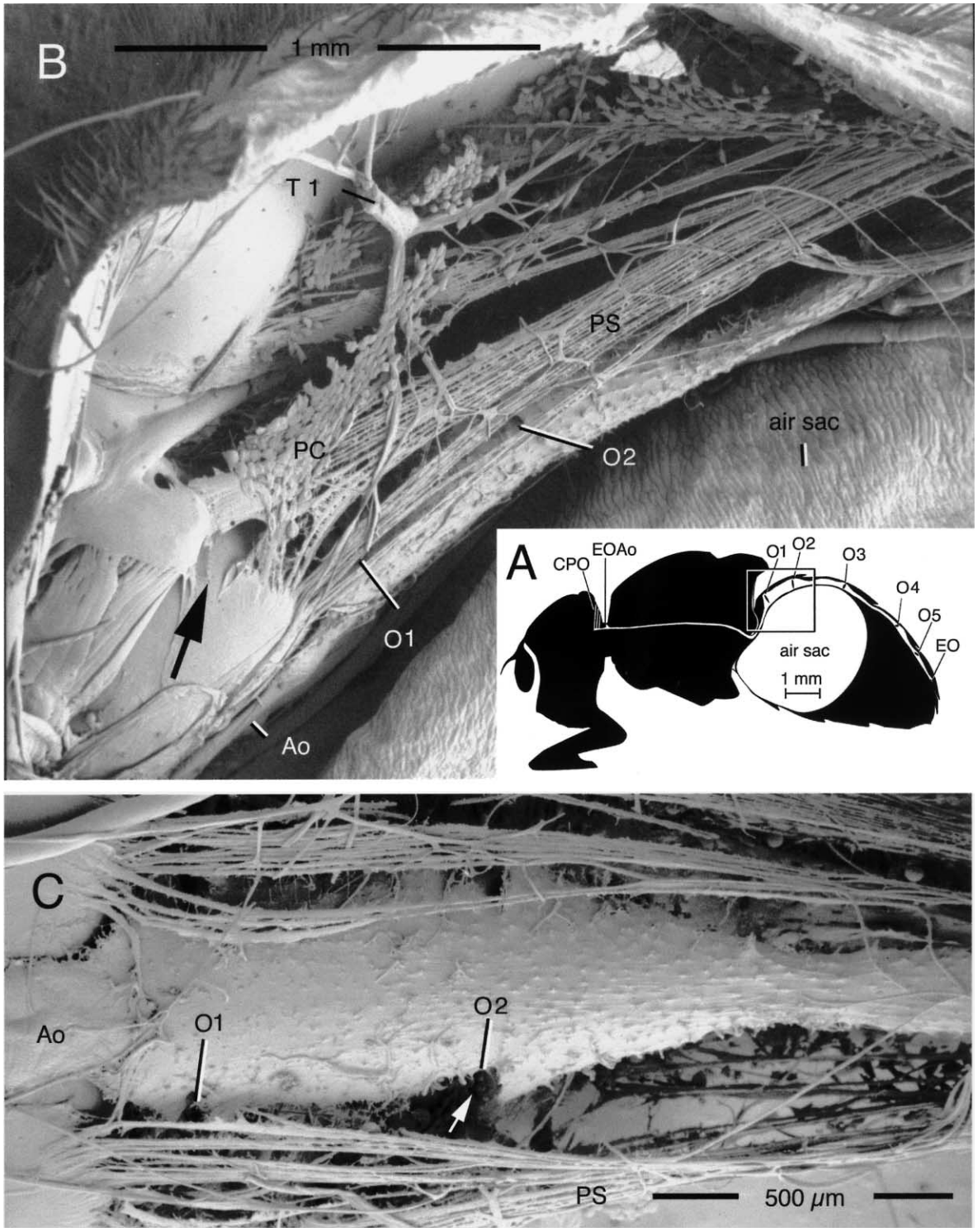

Fig. 1. Orientation and proportions of the dorsal vessel. (a) Survey with complete number and position of ostia, caudal and cephalic openings and position of the large abdominal air sacs. CPO = cephalic pulsatile organ; O1–O5 = incurrent ostia of abdominal segments 1–5; EO = caudal excurrent opening; EOAo = excurrent opening of aorta. (b,c) Heart and pericardial septum in situ in the anterior abdomen. SEM. (b) Lateral view, showing longitudinal muscle fibres of the pericardial septum (PS) with the main inflow passage for aspiration of hemolymph (arrow) from the thorax. The enlarged heart chamber is situated anteriorly to the large air sacs. Left air sac removed. The loose arrangement of pericardial muscle fibres is due to shrinkage. (c) Ventral view at the enlarged anterior heart chamber with anteriorly directed second pair of ostia (O2). T1 = dorsal tracheal trunk of first abdominal segment; O1 = anterior ostium; PC = pericardial cells on the alary muscles.

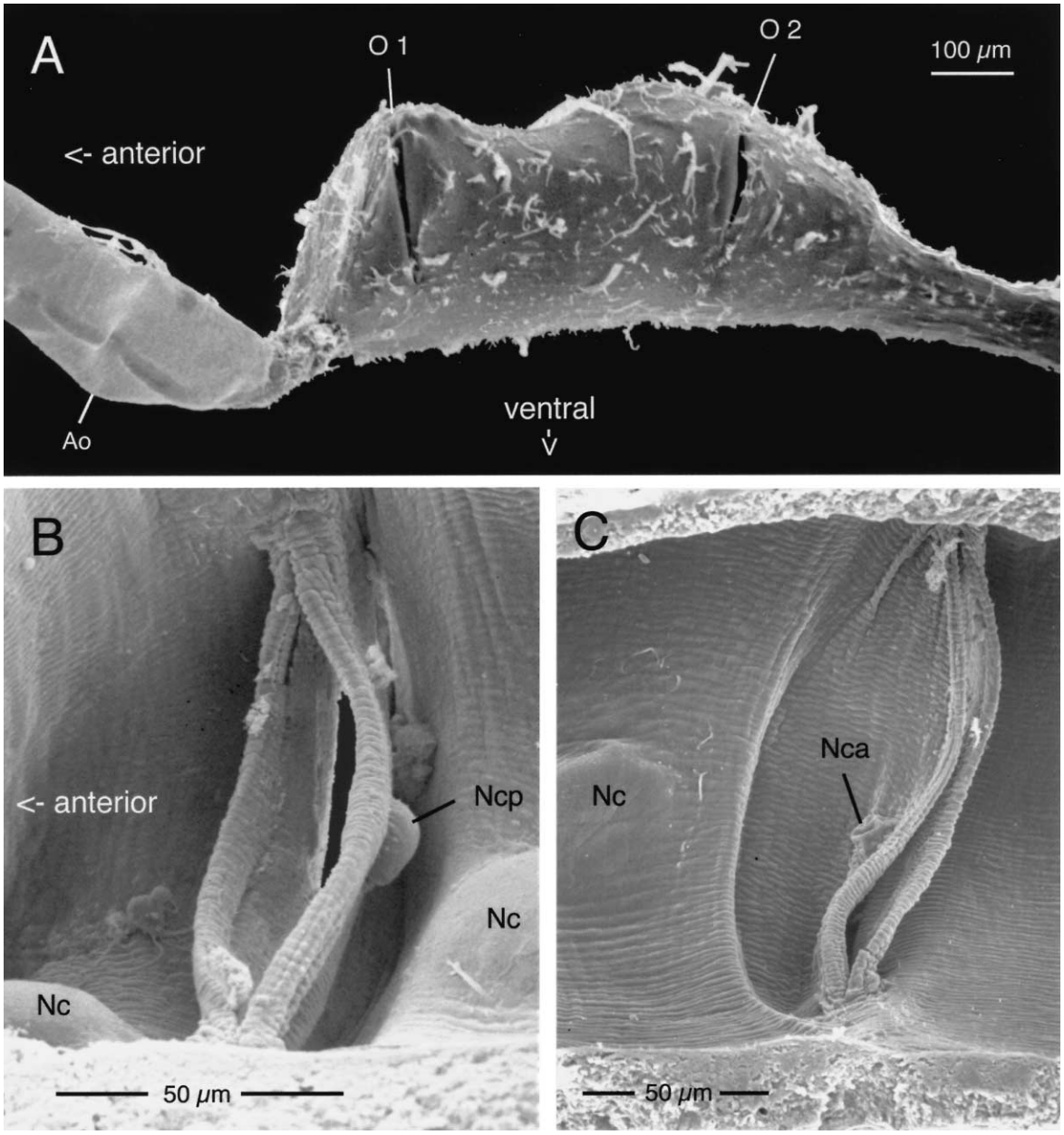

Fig. 2. Isolated anterior heart. SEM. (a) Enlarged heart chamber with two pairs of incurrent ostia (O1, O2) and posterior part of aorta (Ao). (b) First ostium with two ostial lips (cells) protruding into the heart lumen. (c) Second ostium with both ostial lips oriented backward. Nc = nucleus of heart wall cell; Nca = nucleus of anterior ostial cell; Ncp = nucleus of posterior ostial cell. Same orientation in a–c.

containing elastic fibres (Figs 4a–c). Trabecular extensions of this surface layer connect the heart wall with the longitudinal muscles of the pericardial septum (Fig. 4c) and indirectly with the dorsal integument.

3.2. Posterior heart tube

The posterior heart tube is conspicuously narrowed. The diameter of the connecting portion between the anterior chamber and the posterior heart segments and also between the latter segments is only about 100 μm. At the level of the third and fourth pairs of ostia, the diameter increases (120 and 150 μm, respectively) and tapers to 80–100 μm at the level of the fifth segment. The ostia of the third and fourth segment are constructed like those of the anterior chamber. The slits have a vertical orientation relative to the heart axis (Fig. 5a,b). The ostia of the fifth heart segment have generally been overlooked, probably because the ostial slit is oriented more horizontally and therefore almost hidden under the longitudinal muscles of the pericardial septum (Fig. 5c). Near its dorsal attachment site at the pygidium, the caudal end of the heart is troughlike and its lumen is directly facing the hypodermis of the integument. Between the muscle

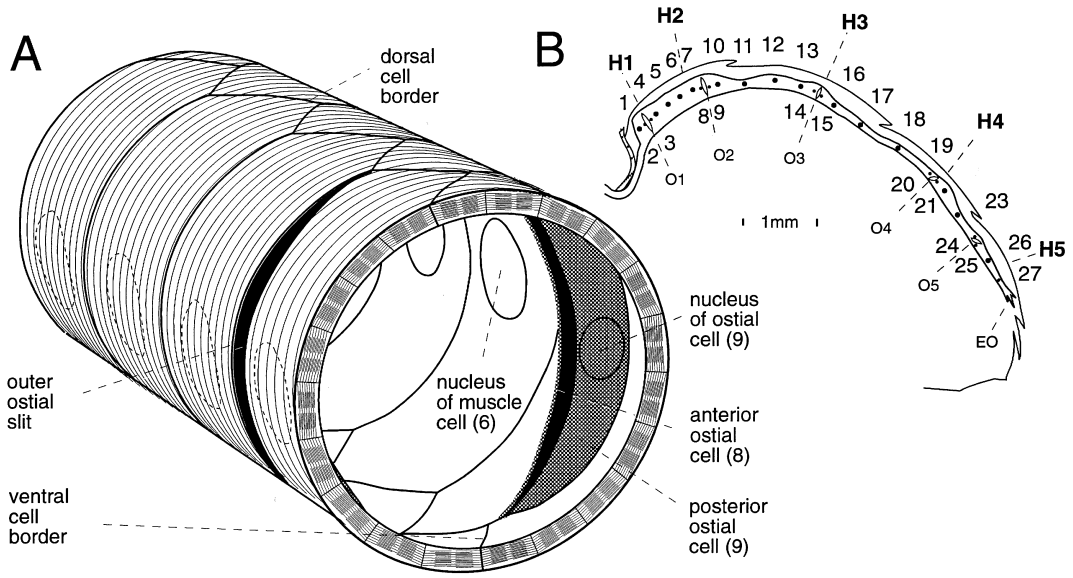

Fig. 3. Cellular arrangement of the heart. (a) Second segment with spirally oriented muscle fibrils. The heart is constructed of two rows of hexagonal cells which contact dorsally and ventrally. A pair of ostial cells (8 and 9) is interspersed in consecuting wall cells. (b) Heart with numbered cell-pairs of wall (upper series) and ostia (lower series). The dots represent the nuclei of the heart cells. The slits (O1–O5) represent the ostia of the first (H1) to the fifth heart segment (H5).

strands attaching to the pygidium, simple spaces are left free, forming two terminal funnel-like excurrent openings and two less obvious subterminal openings (Fig. 5d). They have no valve cells like the incurrent ostia.

The wall of the three posterior heart segments is much thicker than that of the anterior heart chamber. At the level of cell number 16, it is 20–40 μm (Fig. 6a). The myocardium contains a higher portion of muscle fibres and the sarcoplasm is densely packed with mitochondria and glycogen and is equipped with numerous tracheae and tracheoles (Figs 6b,c). The sacroplasm around the nuclei bulges into the heart lumen. The sarcoplasm of pair number 17, forms a mighty swelling along 50 μm (Fig. 6d). It is much more extended than the nucleus. The sarcoplasm of the swellings is less electrondense containing only scattered mitochondria and fewer glycogen granules (Fig. 6e). In whole mount preparations under phase contrast illumination, the region along the total cell length (about 400 μm) looked more dense. When the heart contracts, the cushion-like evaginations are pressed against the opposing heart wall and probably impede a hemolymph passage.

The pericardial septum forms a dense basket of longitudinal muscles around the heart, that forms a distinct pericardial cavity around the anterior heart chamber including its dorsal side. The heart is connected to the pericardial muscle cells and the pericardial muscle cells are interconnected by elastic connective tissue strands (Fig. 1c). The electron dense fibrils (Fig. 4c,e,f) correspond to the elastic fibres, analysed in the connective tissue of the hearts and nerves of the lepidopteran *Calpodes* (Locke and Huie, 1972). At the level of heart segments 3 and 4, typical alary muscles converge laterally and attach at the cuticle. Most of the lateral alary muscles are covered by pericardial cells. At the fifth segment, the longitudinal muscle cells of the pericardial septum are more tightened to the heart wall and the alary muscles are oriented caudally attaching at the pygidial cuticle hidden under fat cells. The pericardial sinus around the anterior heart chamber is connected to the hemocoel outside the pericardial septum via a distinct inflow passage at the anterior attachment site of the pericardial septum (Fig. 1b, arrow). The reflux of the thoracic hemolymph into the large heart chamber is probably facilitated by this opening. The outer slit of the first ostium is directed laterally and close to this inflow passage, the second ostium is directed anteriorly (Fig. 1c, arrow), its inner lips are directed posteriorly (Fig. 2c).

3.3. Thoraco-abdominal aorta

The thoraco-abdominal aorta differs from the heart in many structural aspects. It has a smooth surface (Figs 1, 2a) without trabecular strands for attachment and is not enclosed in a pericardial muscle basket. In the waist region it is depress and adheres directly to the rear of the metathoracic phragma. In the pterothorax, it has a cross shape of about 50 μm. The relaxed wall is only 2–4 μm thick. It consists of four pairs of cells in the thorax, and one pair in the abdomen. The spirally oriented muscle fibres are loosely arranged in an extracellular matrix of connective tissue (Figs 7c,d). In electronmicrographs the less electrondense matrix contains a mesh network of connective tissue fibres. The matrix is coated by basement

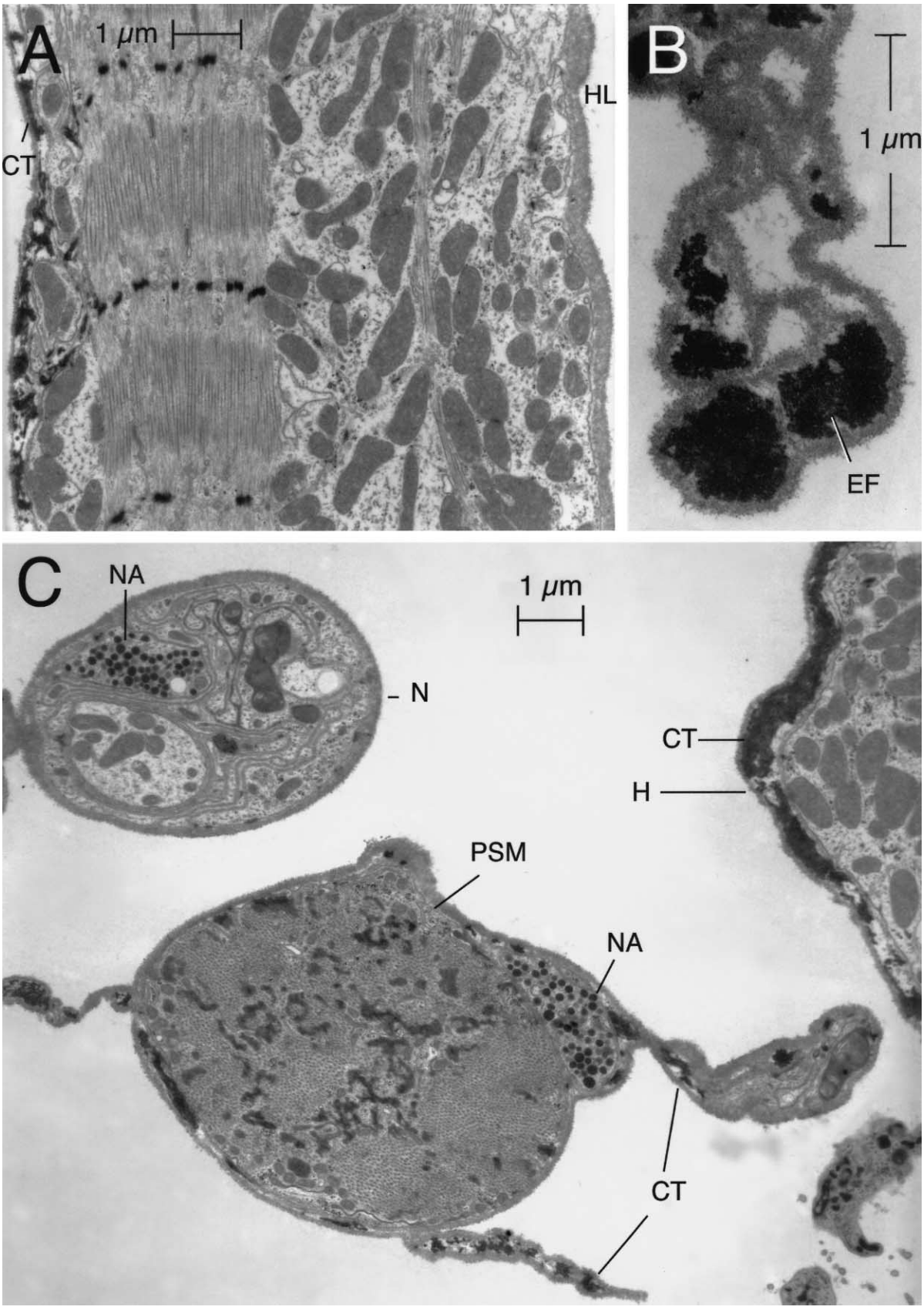

Fig. 4. Muscle cells of anterior heart wall and pericardial septum. TEM. (a) Cross-section of large heart chamber with spirally oriented muscle fibrils. The lumen face (HL) is lined by dense basement membrane, the hemocoel face is covered with basement membrane containing bundles of elastic fibers (CT). (b) Cross-section of extracellular connective tissue strand connecting heart with surface layer of the pericardial muscle cells. (c) Cross-section of pericardial septum muscle (PSM) and heart nerve (N) with neurosecretory axons (NA). CT = connective tissue; EF = elastic fibers; H = heart; HL = heart lumen.

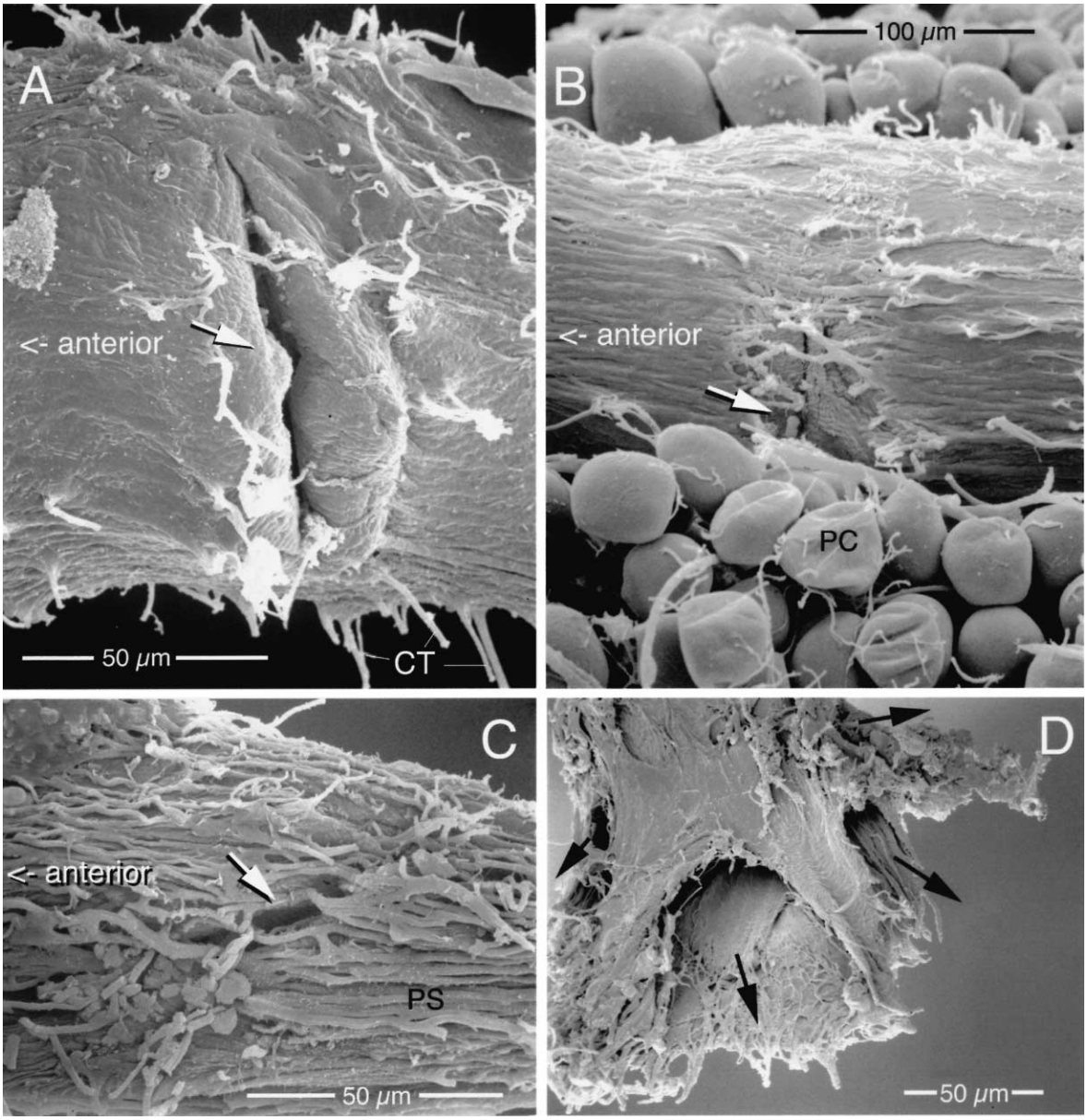

Fig. 5. Posterior heart portion. SEM. (a) Lateral view of 3rd heart segment. (b) Ventral view of fourth heart segment. (c) Lateral view of fifth heart segment, covered by longitudinal muscles of pericardial septum (PS). (d) Dorsal view of posterior heart end with paired excurrent openings. Arrows show presumed outflow direction. CT = trabeculae of connective tissue; PC = pericardial cell.

membranes at both the hemocoel and lumen face. In the prothorax, the orientation of the muscle fibres changes from spiral to longitudinal and the aorta wall is folded (Figs 7a,b). The aorta morphology at the level of the prothorax is complicated by its association with corpora cardiaca complex and the 'proventricular' gut. At the level of the corpora cardiaca neurohema1 axons form part of the wall and their terminals discharge directly into the aortal lumen. At the transition to the neck, the aortal wall is coated by air sacs (Fig. 7a), thus being constructed of two cell layers.

In a fly with a ventrally fenestrated anterior abdomen, deprived of the large air sacs and intestine, the transitional area of heart chamber and abdominal aorta was directly observed. The different histological nature of the aorta is obvious by its clear and smooth surface in contrast to the opaque and structured wall of the heart plus pericardial sheath. A different behaviour in pulse propagation is also visible in these preparations: When the heart rapidly contracts, the neighbouring part of the abdominal portion of the aorta rapidly dilates and the aorta is passively extended by the presystolic pressure wave preceding the systole of the heart. The aorta contraction following the dilation looks like a slow relaxation or collapse rather than a systole. In the preparations during backward-pulses, the abdominal part of the aorta remained relaxed

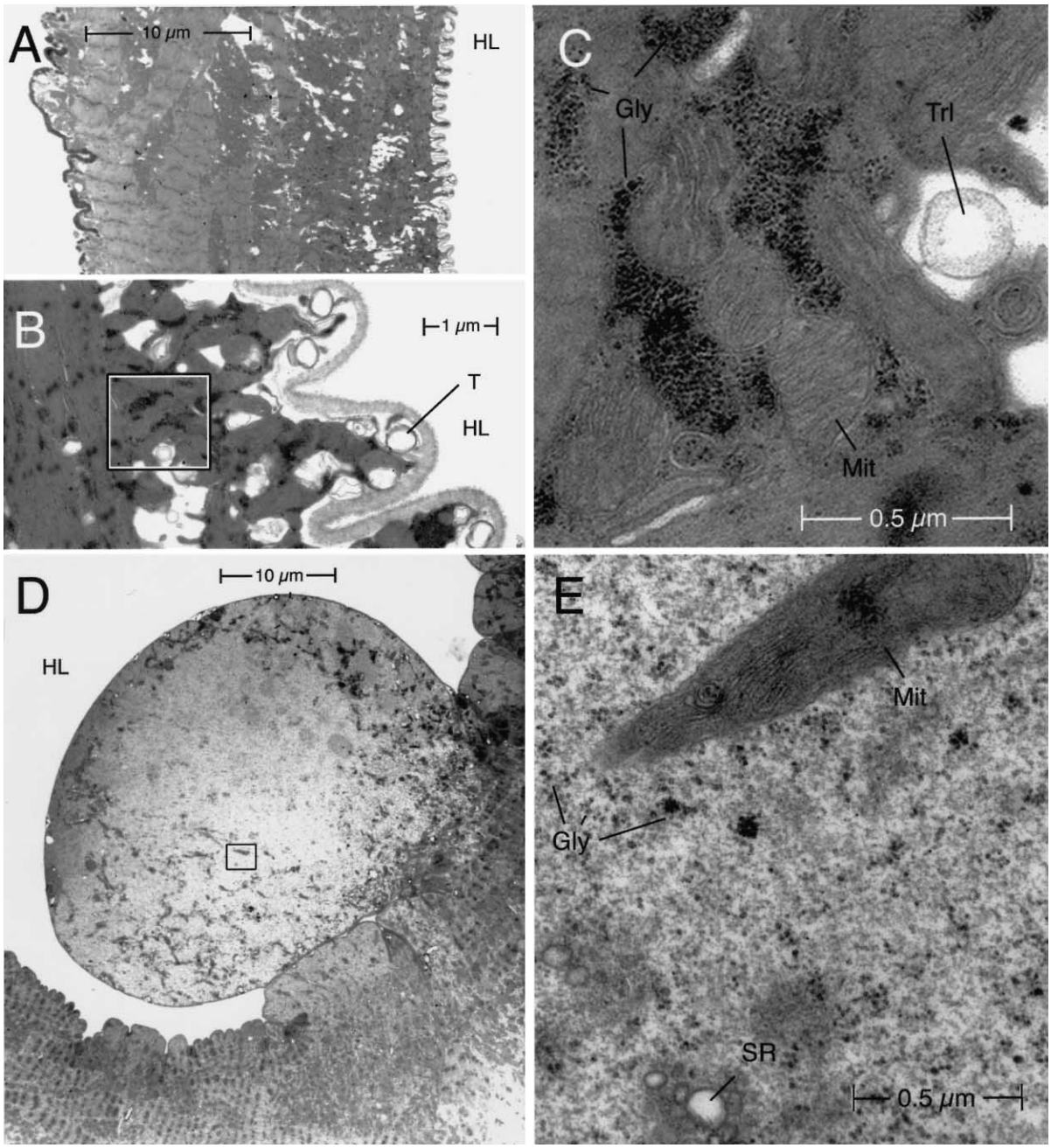

Fig. 6. Cross-sections of the myocardium at the level between third and fourth ostium. TEM. (a) The heart wall is much thicker and contains more muscle fibres than that of the anterior heart chamber. (b) The dorsal part of cell 16 with unusually rich tracheal supply at the lumen face. (c) Detail from b. The sarcoplasm is densely packed with mitochondria (Mit), glycogen (GLy) and tracheoles (Trl). (d) Sarcoplasmic swelling at the level of cell 17 narrowing the heart lumen. (e) Detail from d. The swelling contains scattered glycogen, mitochondia and sarcoplasmic reticulum (SR).

or moved only weakly, not obviously dilated by a pressure wave!

The aorta has no ostia. The single (excurrent) opening terminates already in the middle of the highly flexible neck, not in the head. At the opening, the aorta is composed of a dorsal cuticular 'roof' and of a ventral 'floor' of longitudinal muscles (Figs 8, 9). The cuticular roof arises from complex infoldings of the dorsal and lateral neck integument and its endocuticle interdigitates laterally with connective tissue cells of the aorta muscle floor (Figs 8e–g, 9b). In the lateral transition zone, connective tissue cells mediate between integumental cells and muscle cells. They have a variable amount of microtubules. Few of them are densely packed with microtubules (Fig. 9c,d). The cell processes are laterally interconnected by septate junctions and terminally their microtubules attach to electrondense plaques at the endocuticle (Fig. 9c). Towards the posterior neck, the ventral aortal cell

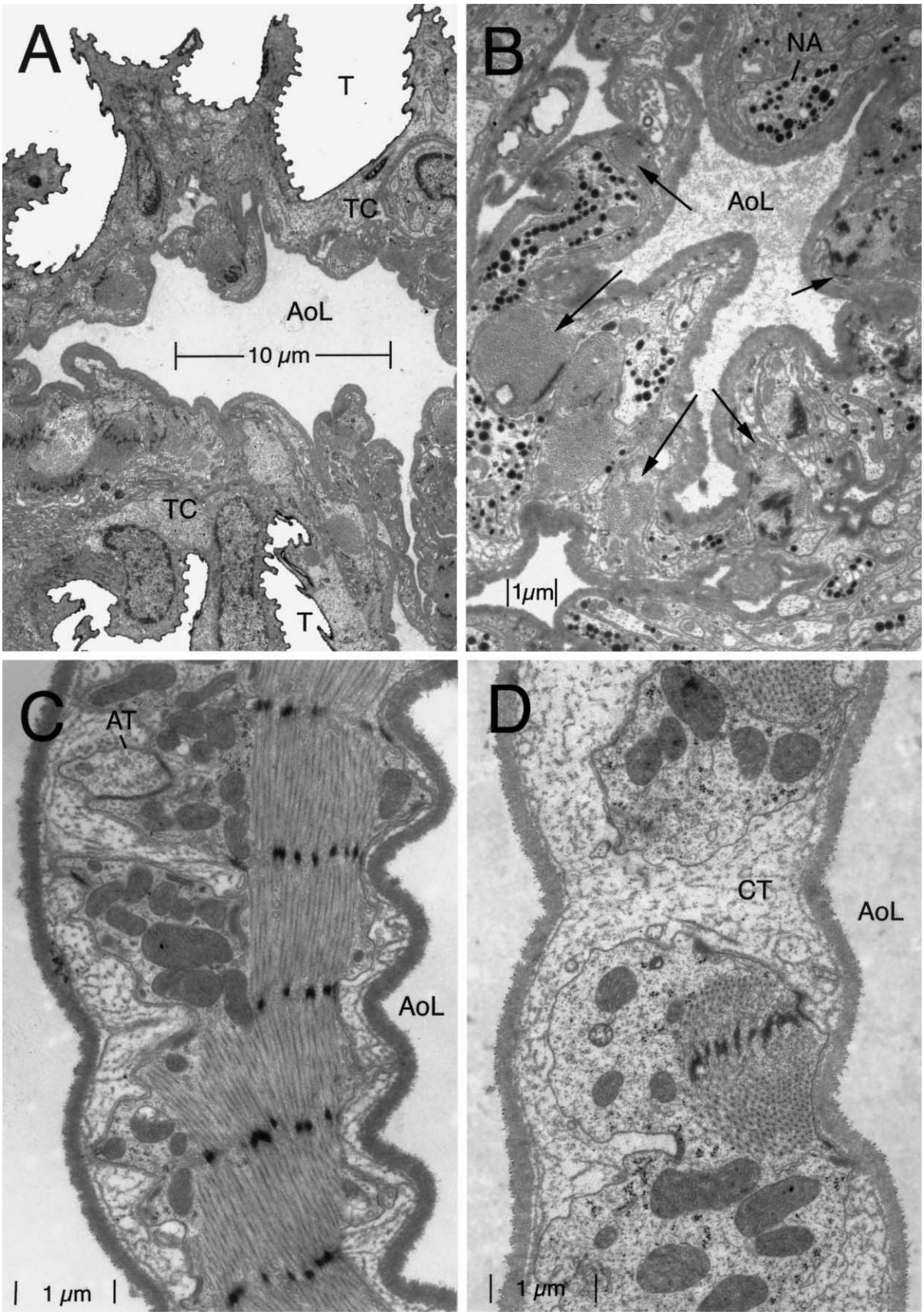

Fig. 7. Cross-sections of thoracic aorta. TEM. (a) Anterior part in the prothorax. The folded aorta consists of an inner layer of longitudinal muscle cells and an outer layer of tracheal cells (TC). It is entirely surrounded by tracheal air sacs (T). (b) Aorta at the level of the corpora cardiaca consisting mainly of neurosecretory axon terminals (NA) and few scattered longitudinal muscle fibres (arrows). (c) Mesothoracic aorta wall with spirally oriented muscle fibres. AT = synapsing axon terminal. (d) Mesothoracic aorta with obliquely sectioned muscle cells. They are embedded in an extracellular matrix with a meshy network (CT). AoL = lumen of aorta.

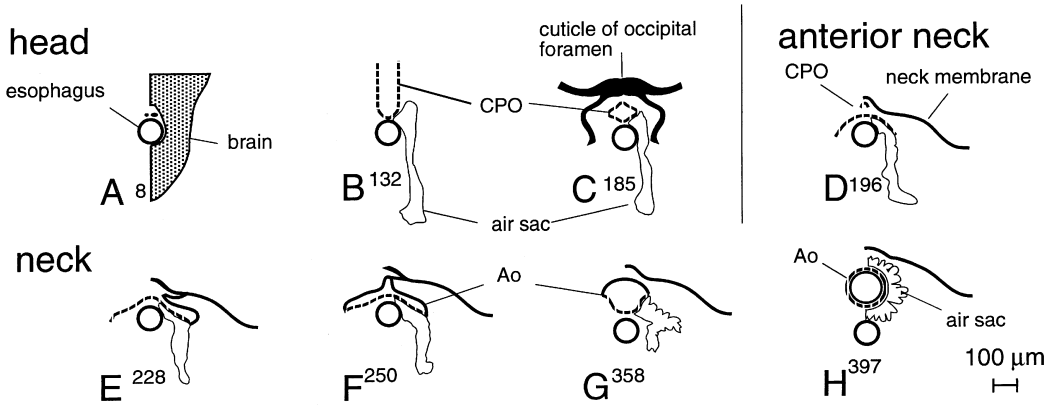

Fig. 8. Line drawings of cross-sections of the cephalic pulsatile organ (CPO) in the posterior head and of the aorta in the neck (semischematic). They belong to a series of 397 sections used for the 3-D-reconstruction of Fig. 12 and serve for orientation of the TEM-figures. Numbers correspond to the original sections. (a) Anterior region with CPO muscle connections passing between brain and esophagus toward the front. (b) U-shaped CPO with suspending muscle strands. (c) Muscular tube of the CPO at the level of the occipital foramen. (d) CPO in the anterior neck is divided into a dorsal muscle portion fixed at the neck membrane and a ventral muscle plate. (e) The ventral muscle plate is joined by a cuticular fold from the lateral neck membrane. (f) The cuticular folds of both sides are united and form the 'roof' of the aorta while the muscular plate forms the 'floor'. (g) The aorta is separated from the neck membrane but still consists of a cuticular roof and a muscular floor. (h) At the posterior neck the cuticular component of the aorta is internalised facing its lumen, while the muscle and connective tissue cells form the outer sheath of the aorta. In the sections a–g the air sac is attached to the ventral face of the muscle membrane. In h the air sac surrounds the aorta almost entirely.

layer encircles the dorsolateral cuticle, while the muscle cells fuse outside dorsally, the cuticle layer fuses inside ventrally and changes into an unconspicuous thin cuticle which is continuous with the basement membrane of the thoracic lumen face (Figs 8h, 9e).

3.4. Cephalic pulsatile organ

The cephalic pulsatile organ (CPO) consists of a u-shaped plate of longitudinal muscles with dorsal muscle strands attaching along the median occipital ridge of the head (Figs 8b, 10a, 11d, 12). At its anterior end, the plate converges to a massive muscle strand, which runs above the esophagus through the brain towards the front (Figs 8a, 11a). At its posterior end the plate forms a short tube which passes through the occipital foramen (Figs 8c, 10b, 12a). In the neck this muscle tube divides into a converging strand which attaches to the dorsal neck membrane and a ventral diverging tray, which is continuous with the enlarged ventral muscular membrane of the aorta opening (Figs 8d, 12). Thus, the CPO has a posterior (incurrent) opening in the anterior neck opposite to the aorta opening and an (excurrent) opening on the rear of the brain. The underside and lateral parts of the entire CPO plate and of the aorta muscle membrane are fused with the longitudinal tracheal air sacs. These are part of the air sacs which surround the prothoracic aorta and communicate with the anterior thoracic spiracles.

In contrast to the massive anterior muscle strands the CPO muscle membrane and the dorsal muscle strands consist of muscle cells embedded into a less electrondense extracellular matrix of connective tissue (Fig. 11b,d). Its lacune-like character may partly be due to suboptimal fixation.

4. Physiological results

The heart of intact and unnarcotized *Calliphora* exhibits a very regular rhythm of longer pulse periods, alternating with shorter pulse periods. There is no difference in tethered and untethered flies. When locally applying a slight temperature excess to the heart by a laser beam between the second and third heart segment, alternating heating and cooling temperature changes are recorded by thermistors (T-method, Fig. 13). The temperature curves on both thermistor sites show a reciprocal effect. At the anterior site (at H2) the temperature increases during the longer pulse periods, at the posterior site (at H3) the temperature increases during the shorter pulse periods. As the temperature rise indicates that the hemolymph pulses must come from the heating site, it can clearly be deduced, that in the intact fly the longer pulse periods represent the forward-pulse periods, the shorter ones represent the backward-pulse periods.

When the thermistors are heated to an excess temperature of 1.5–1.8°C above that of the resting insect with its body temperature being at ambient, each heart pulse produces a cooling effect upon the site below the measuring thermistor. This method allows the use of convective effects for pulse recording (C-method). The mean pulse rate of the forward-pulse period is 3.08 ± 0.74 pulses s^{-1} at 20°C ($N = 3$ flies and 72 periods, range = 2.4–

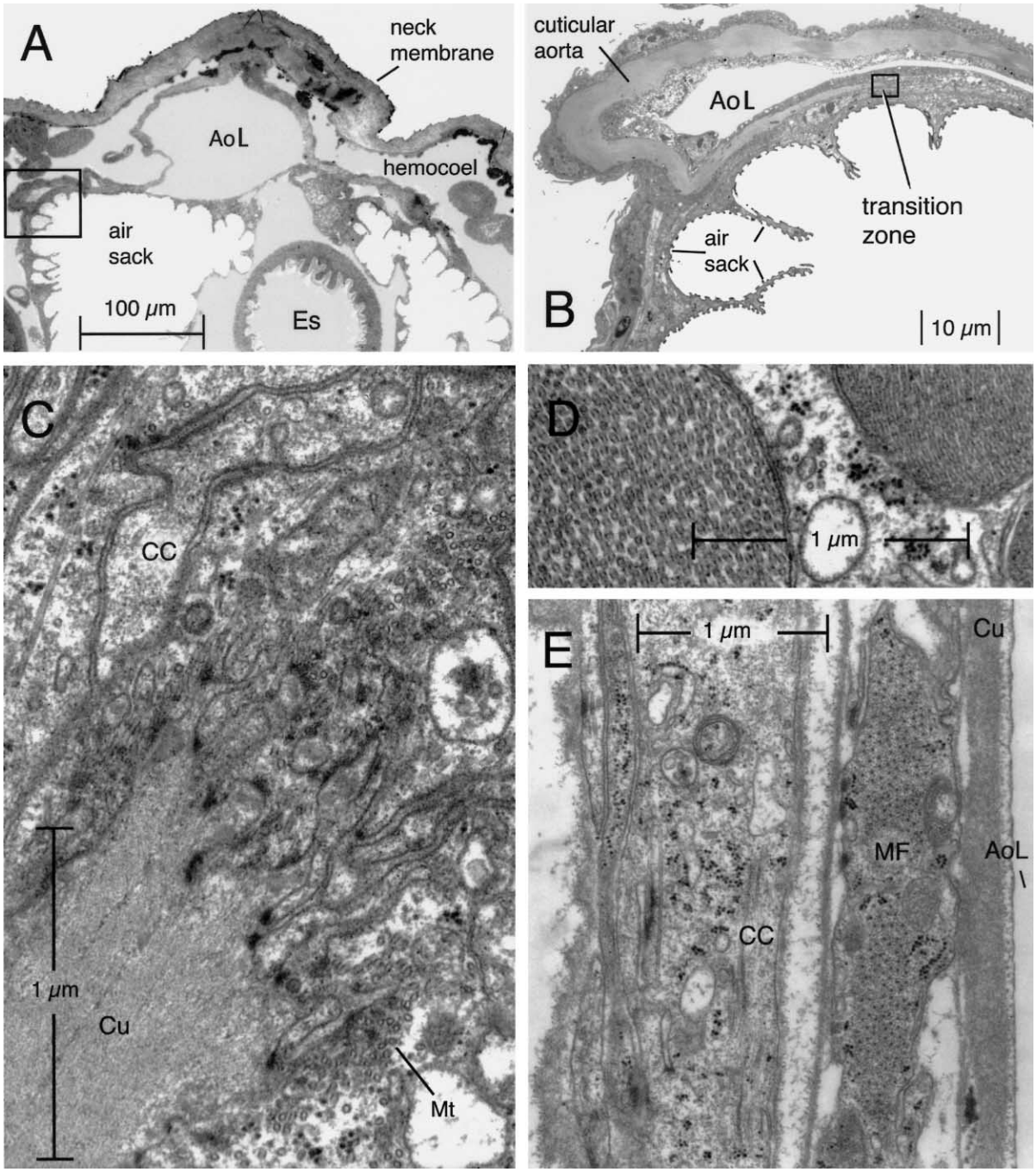

Fig. 9. Cross-sections of aorta in the neck. TEM. (a) Cuticular aorta 'roof' originating from the folded neck membrane. See Fig. 8f. (b) Detail from a. Lateral region of aorta with transition of cuticular 'roof' to basal muscular membrane. (c) Connection between cuticular aorta (Cu) and connective tissue cells (CC) of basal muscular aorta by interdigitations and microtubule (Mt) attachment sites. Detail from b. (d) Processes of connective tissue cells with different densities of microtubule arrangement. Most cells have only scattered microtubules as in the centre. The cells with dense microtubule equipment accompany only the lateral cuticular aorta in the transition zone. (e) Aorta wall at the posterior neck. The dorsal cuticle is displaced into the lumen of the aorta while the muscle cells and connective tissue cells encompass it. See Fig. 8h. Orientation of the muscle fibres (MF) is longitudinal, of the microtubules of the connective tissue cell (CC) is transversal. AoL = lumen of aorta.

4.1), the mean frequency of the backward-pulse period is 4.67 ± 0.82 pulses s^{-1} at 20°C ($N = 3$; $n = 72$, range = 3.6–5.6). With the C-method in addition, changes in thermal conduction give information about local hemolymph accumulation or reduction, which is often a consequence of discontinuous hemolymph transport. At the second and third heart segments, the short periods with the backward pulses lead to a drastic overall temperature decrease, whereas in the course of the forward-pulse periods an exponential rewarming occurs (Fig. 14:

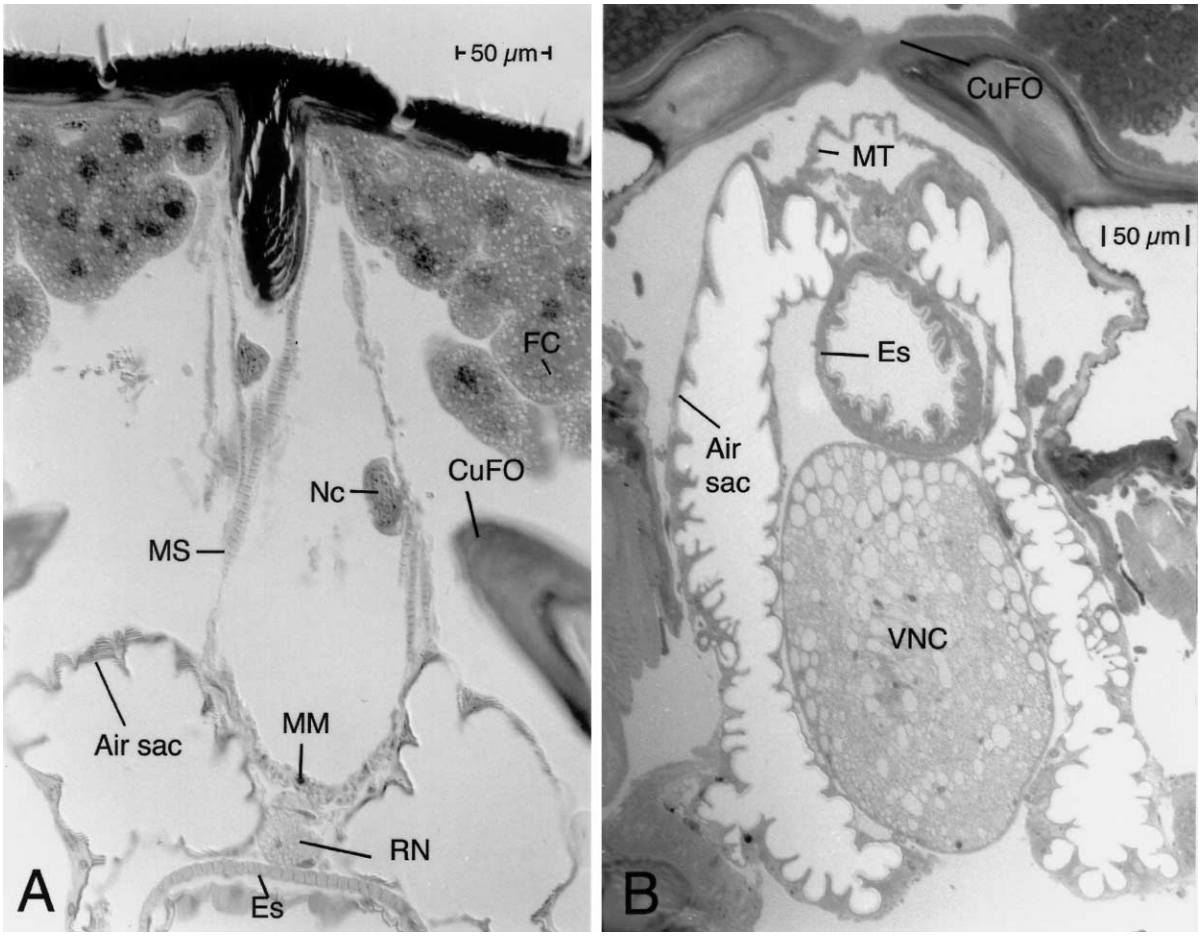

Fig. 10. Cross-sections of the cephalic pulsatile organ (CPO). Photomicrographs of semithin sections. (a) U-shaped muscular membrane (MM) with attached air sacs and muscle strands (MS) fixed besides the median ridge of the occipital cranium. Section corresponds to Fig. 8b. (b) Muscular tube of the CPO (MT) passing through the occipital foramen of the head. Compare Fig. 8c. Nc = nucleus of suspending muscles bulging into the hemocoel; CuFO = cuticle of foramen occipitale; Es = esophagus; FC = fat cell; RN = recurrent nerve; VNC = ventral nerve chord.

H2). These conductive changes reflect the compensatory volume changes of the large air sacs around the anterior heart.

C-measurements at the occipital cranium were originally intended to measure the pulse effects of the aorta, which was assumed to be situated in the posterior head. A crucial point was the question, whether hemolymph is aspired by the aorta opening during backward beating of the heart. The C-curves from the rear of the head revealed an intermittent pulse periodicity that is coordinated with the heartbeat periodicity but not with the pulse rate: During forward-pulse periods strong pulses with a rate of $1.8 \pm 0.51\ s^{-1}$ were recorded (N = same three flies as above, $n = 48$ periods, range = 0.5–2.6). This rate is significantly lower than that of the heart. During backward-pulse periods of the heart no pulses could be detected here at all, but instead a long pause with a rewarming occurred (Fig. 14: CPO). This must be interpreted as a decrease in conductive cooling of the heated thermistor site and suggests that hemolymph volume of the head is reduced by backward beating and air sac volume increases for its compensation. These data originally stimulated the detailed investigation of the CPO and measurements of the intratracheal pressure.

Simultaneous measurements of the intratracheal pressure at the anterior thoracic spiracle show that positive pressure pulses of up to 7 Pa coincide with the CPO-pulses. In contrast, during backward beating of the heart, almost no pulses are detectable but a more continuous negative pressure of $-3 - 5$ Pa arises in the prothoracic tracheal system (Fig. 14: SpI).

5. Discussion

5.1. Periodic hemolymph shift between anterior body and posterior abdomen by heartbeat reversal

Anterior and posterior hemocoel in *Calliphora* are separated by a pair of large abdominal air sacs which are

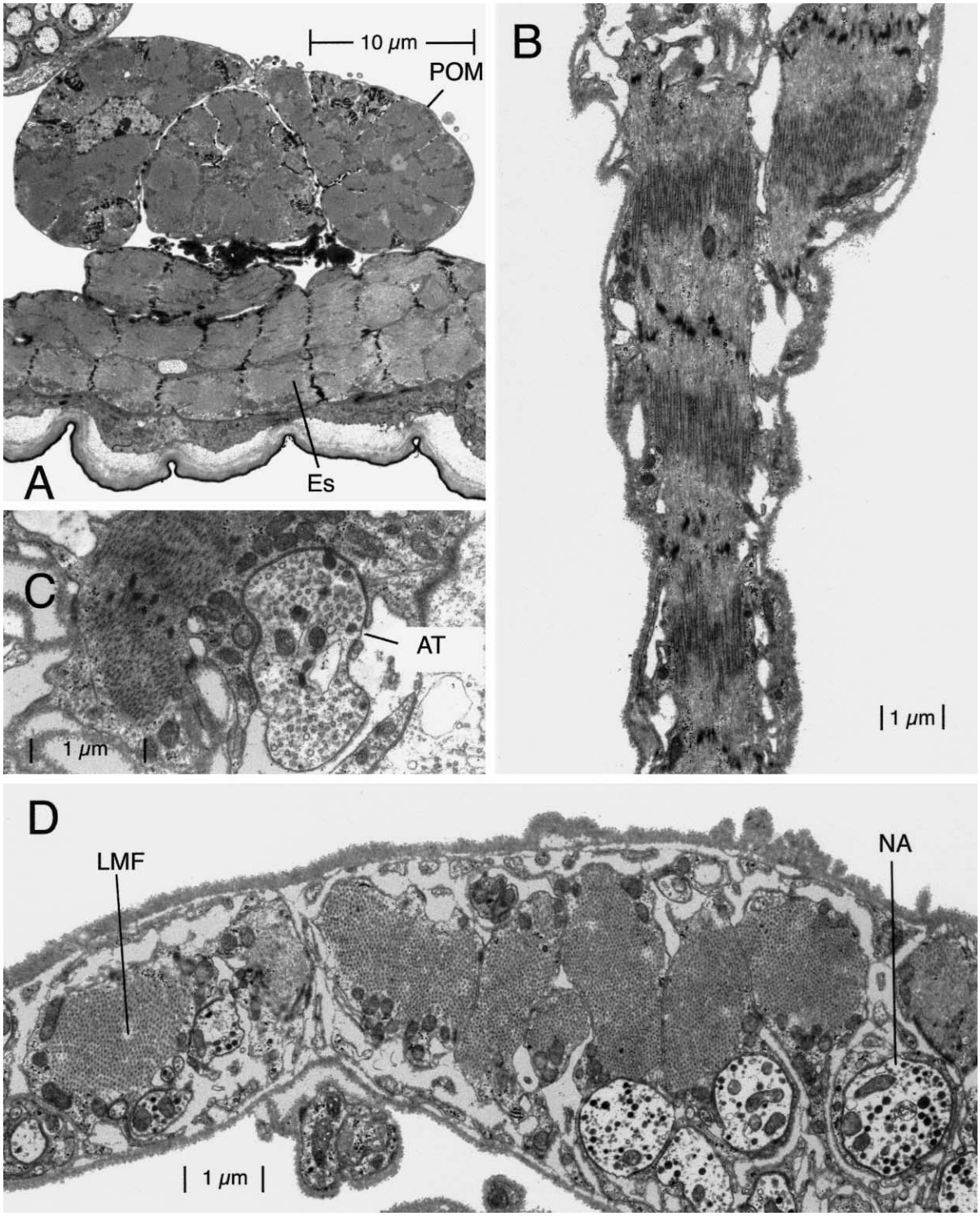

Fig. 11. Muscle cells of the cephalic pulsatile organ. TEM. (a) Cross-section of the massive muscle fibres (POM) passing through the brain towards the frons above the esophagus (Es). Compare Fig. 8a. (b) Longitudinal section of suspending muscle. Compare Fig. 8b. (c) Detail from a suspending muscle with synapsing axon terminal (AT). (d) Cross-section of CPO-membrane with longitudinal muscle fibres (LMF) and neurosecretory axons (NA).

functioning like a septum. As in other higher flies, *Calliphora* lacks a ventral diaphragm and the perineural sinus in the abdomen (Richards, 1963). The dorsal vessel is the only connection between both hemocoel partitions. It is important that the large anterior heart chamber is situated in front of these air sacs. The heart periodically

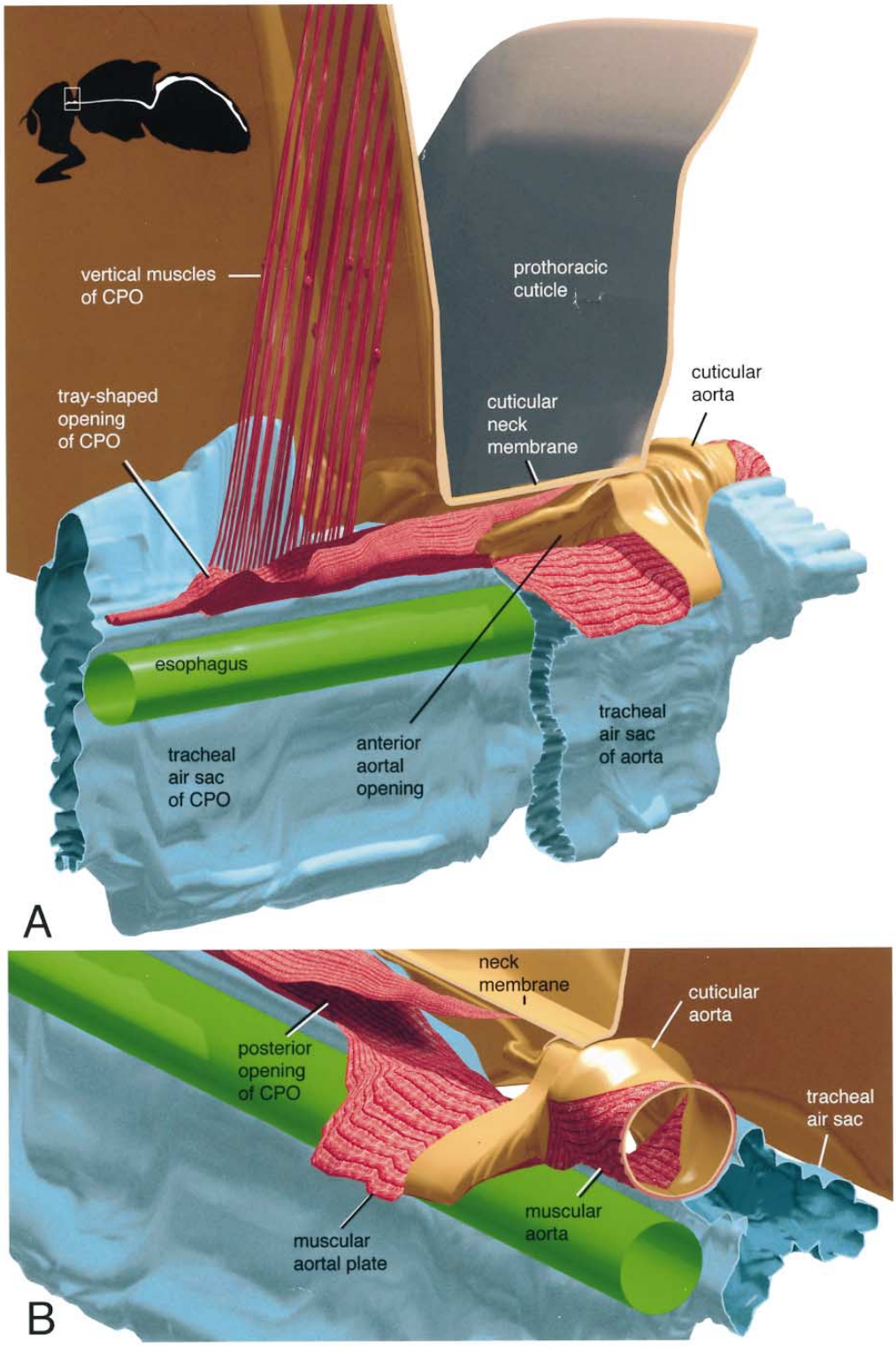

Fig. 12. CAD-reconstruction of the cephalic pulsatile organ (CPO) and aorta in the posterior head and neck. The nervous system, left integument, left suspending muscle strands of the CPO and left air sac partly omitted. (a) View (obliquely from anterior) at the anterior openings of the CPO and of the aorta end with the transitional zone of the enlarged muscle plate. (b) View (obliquely from behind) at the posterior opening of the CPO and the cut aorta.

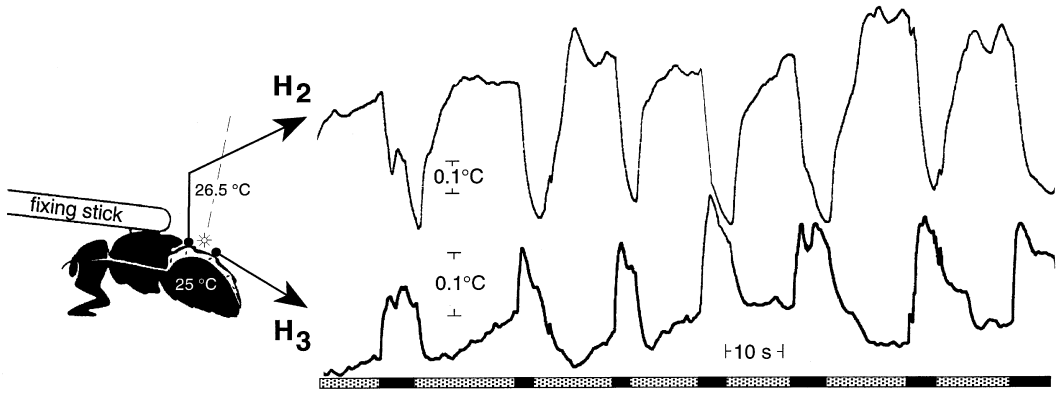

Fig. 13. Periodic heartbeat reversals and determination of heartbeat direction by temperature measurements (T-method) on the second (H2) and third (H3) heart segment under application of heat via laser beam (symbol) between these segments. The heating effects (temperature rise) are reciprocal at both thermistor sites. Rise at the anterior site indicates forward beating, rise at the posterior site indicates backward beating. The longer periods (dotted bars) are forward, the shorter periods (black bars) are backward.

reverses its transport direction for hemolymph exchange between anterior body and posterior abdomen. As in Lepidoptera and Coleoptera, the partitioning of anterior and posterior hemocoel, combined with the oscillating hemolymph transport, allows economic use of a small hemolymph volume for tracheal ventilation in the lightweight flight adapted adults (Wasserthal, 1975a,b, 1981, 1982a,b, 1996).

In *Calliphora*, periodic changes of heartbeat frequency have been recorded electrophysiologically, but heartbeat reversal was either never seen (Normann, 1972, Duve et al., 1993) or was interpreted in different ways.

With the heat marking technique—heating the hemolymph between two heart segments—a clear response regarding pulse direction could be given. The reciprocal heating effect of both measuring sites demonstrates that the shorter periods with the higher pulse rate are backward, the longer pulse periods with the lower pulse rate are forward, confirming older results using a similar contact-thermographic technique but under heating the thorax and measuring with one thermistor at the abdominal heart (Wasserthal 1982b). The data from intact flies contradicts that in former publications (Brazeau and Campan, 1970; Queinnec and Campan, 1975; Thon and Queinnec, 1976; Thon, 1980, 1982) and a recent interpretation of electrophysiological data from semi-isolated heart preparations of *Phormia* flies (Angioy and Pietra, 1995). On the basis of the metachrony of a single pulse recorded from the aorta and the first heart segment, the authors concluded, that the 'slow phases' represent the backward pulses, while from the metachrony of a single documented pulse from a different constellation of measuring sites (H1 and H3) the forward pulses were attributed to the 'fast phase'. As the aorta probably transports no hemolymph during backward beating at all and pulses of the cephalic PO are only performed during forward pulses of the heart with an independent pulse rate (Fig. 14 and see below), a comparison of heartbeat metachrony should be based on recordings from the same couple of (abdominal) heart segments for both beating directions. In addition, heartbeat frequency is said to differ greatly between intact flies and semi-isolated heart preparations (Normann, 1972).

5.2. A central heart chamber for maintenance of a lateral circulation within the thorax during both forward- and backward-beating

The more detailed analysis of heart anatomy in combination with the physiological data of heartbeat reversal helps to understand the circulatory system of the fly. The enlarged heart chamber in the anterior abdomen with two pairs of incurrent ostia reminds one of the central heart of some other arthropods, such as decapod crustaceans. Its central position anteriorly of the large abdominal air sacs facilitates aspiration of the hemolymph from the anterior body, especially from the thorax during both forward- and backward-pulse periods. Although the hemolymph is shifted between anterior body and posterior abdomen by heartbeat reversal, in the course of the forward-pulse period as well as at the beginning of the backward-pulse period, the hemolymph is available in the thorax for aspiration by the anterior heart chamber. Aspired hemolymph passes along the haltere muscle region in the metathorax and enters through a special opening in the anterior pericardial septum which is close to the first ostia (Fig. 1). The orientation of the outer ostial opening is directed towards this pericardial opening. Thus, a hemolymph circulation can be deduced to occur in the lateral thorax and anterior abdomen throughout most of the forward-pulse period and at the beginning of the backward-pulse period.

The anterior heart chamber with a volume of about 0.14 μl (calculated from mean diameter of 400 μm, length of 1100 μm and a wall of 8–10 μm thickness) is adapted to store most hemolymph volume of each pulse. The

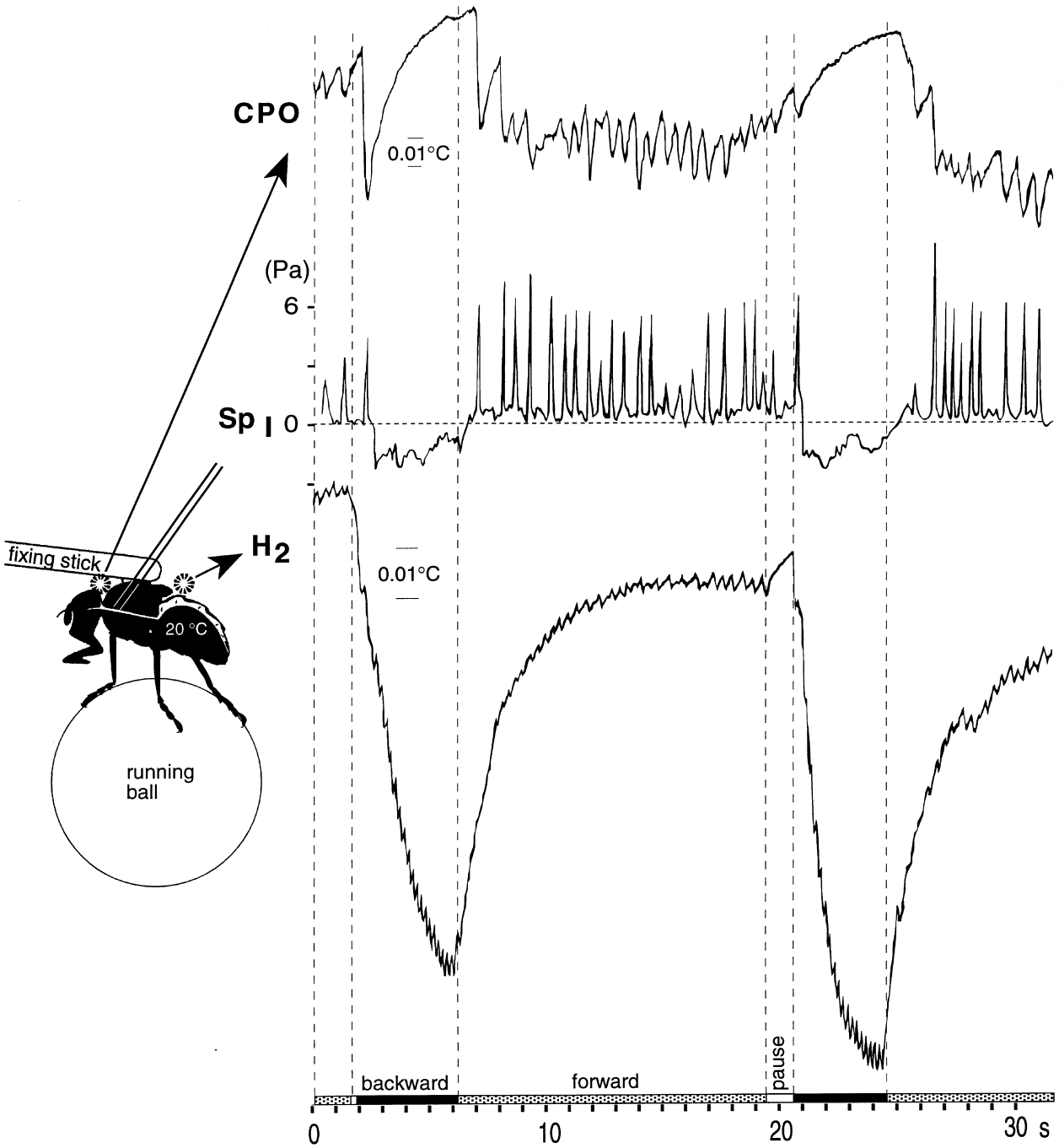

Fig. 14. Periodic heartbeat reversals and coordinated periodicity at the cephalic pulsatile organ (CPO) and their effect upon intratracheal pressure at the prothoracic spiracle (Sp I). Heart pulses (measured on the second heart segment = H2) and CPO-pulses are visualized by their convective cooling effects upon slightly heated thermistor sites (C-method, rosette symbols). The backward-pulses have a higher pulse rate (3.6 Hz), the forward-pulses have a lower pulse rate (2.4 Hz) at 20°C. During forward-pulse periods the CPO pulsates, however with a lower frequency (1.6 Hz) than the heart. During backward-pulse periods the CPO stops beating and the rewarming at the CPO-site indicates reduced overall convection and conduction. The overall cooling during backward beating of H2 indicates an increase of thermal conduction due to hemolymph accumulation in the abdomen. The intratracheal pressure curves at the anterior thoracic spiracle reflect mainly the discontinuous pulse activity of the CPO. The positive pressure pulses are synchronous with the CPO-pulses. The negative rather continuous pressure at SpI during CPO-pause arises from hemolymph reduction by the backward beating heart.

posterior heart tube has a narrow lumen of only 50–100 μm diameter with a solid muscle wall of 2–4-fold (20–40 μm) that of the anterior chamber. It is suggested that the hemolymph is transported from the posterior abdomen into the anterior body only during about the first half of the forward beating period. During the following half of

the forward-pulse period, the posterior heart has to work against the increasing negative pressure of the posterior abdominal hemocoel with reduced hemolymph volume and distended air sacs. The anterior heart chamber then refills easily from the anterior hemocoel with more relaxed air sacs. During this second half of the forward-pulse period, the posterior heart tube has mainly to prevent the backflow of the hemolymph into the posterior abdomen. By its solid wall and narrow lumen with protruding sarcoplasmic swellings at cell pair number 17, a retrograde flow is prevented during forward peristalsis. During backward-pulse periods, the suction force of the anterior heart chamber and its volume supplies each backward-pulse. The posterior heart tube with its narrow lumen and massive muscle layer is ideal for pressing the hemolymph through its caudal excurrent openings. It discharges into the posterior abdominal hemocoel where the negative pressure due to the distended air sacs now helps in retrograde hemolymph transport. It is assumed that after depletion of most hemolymph from the anterior body in the course of the backward-pulse period no more refilling of the anterior chamber is possible: this implies that the backward pulses must stop, and might explain why the backward-pulse periods are shorter than the forward-pulse periods. The higher frequency of the backward pulses might be caused by the more straight and direct route that the backward wave has to follow and its direct passage through the paired caudal openings below the pygidium.

5.3. Effects of aorta morphology on hemolymph transport and its different activity in forward and backward beating

The lower forward frequency of the heart might result from the higher resistance offered by the involvement of the aorta and by several structural characteristics of this vessel: (1) the curved route of the aorta; (2) its complex folded prothoracic region and opening in the narrow neck, and (3) its elastic wall with sparse muscle fibres which probably serve for preventing overstretching rather than contracting. This is also suggested by the fact that the aorta, along most of its surface, is not kept open by pericardial muscles or connective tissue strands in contrast to the heart tube. The aorta is not able to dilate autonomously to fill its lumen with hemolymph, especially at the complex plicated anterior end in the neck with longitudinal muscle fibres. Thus, hemolymph can enter the aorta only by external pressure, which is produced by the heart only during forward pulses. The elastic wall seems to have similar mechanical properties like that of an artery in vertebrates. By smoothing the pressure effects of the heart during forward beating, it operates like a pressure buffer. Thus the dipteran 'aorta' is a morphologically and functionally much different structure than the term 'heart' might suggest.

5.4. An aorta valve mechanism in the neck by air-sac expansion

During backward beating, the anterior heart chamber aspires hemolymph from the anterior body hemocoel including that of the head. Only the neck opening could theoretically be used as an incurrent opening during backward beating of the heart, because no incurrent ostia occur in the thoracic aorta. However, it is indirectly shown by the lack of pulses and the reduction of thermal conduction in the posterior head during backward beating of the heart that no pumping activity of the CPO or neck aorta takes place. Instead, the compensatory volume expansion of the air sacs below the aorta opening must press the ventral aorta muscle membrane against the dorsal cuticular folds of the aorta 'roof'. In the anteriormost part of the prothoracic aorta where the air sacs fully surround the thin wall of the aorta, their expansion should additionally compress the aorta lumen. The negative pressure pulses during the backward pulses of the heart measured at the anterior thoracic spiracles are smoothed (Fig. 14). This also indicates that the aspirating opening of the heart during backward beating is distant from the anterior spiracles.

5.5. The cephalic pulsatile organ (CPO), a new organ for combined hemolymph distribution and tracheal ventilation in the posterior head

While antennal pulsatile organs are described in the anterior head of flies (Miller, 1950; Clements, 1956; Dudel, 1977, 1978), no accessory pulsatile organ is reported from any insect in the posterior head (Pass, 1998). As the opening of the aorta in the neck was overlooked, the opening of the CPO on the rear of the brain was misinterpreted as the aorta opening (Normann, 1972).

The mode of function of the CPO can be explained as follows. The CPO sucks hemolymph into the head and distributes it. By this separate pumping structure, the full hemolymph volume leaving the aorta opening during each forward pulse is split into two streams, one supplying the dorso-lateral head via CPO and the 'excess', which is not aspired by the CPO, entering the neck hemocoel from where it can be distributed either into the ventral head or the thorax.

The main pumping force of the CPO probably comes from the dorsal suspending muscle strands (Fig. 12). When contracting, they lift the tray-shaped longitudinal muscle membrane of the CPO with its attached air sacs. While the lumen between CPO-tray and the occipital integument is reduced during contraction of the CPO muscle strands, the air sacs are stretched dorsally. During relaxation of the CPO muscles the distended air sacs at the underside of the muscle membrane reshorten thus functioning as antagonists to the CPO muscles. The CPO

thus is a bifunctional organ that with the same pulse moves the hemolymph and ventilates the attached air sacs in the head. The strong convective hemolymph pulses—recorded by the thermistors—produced such efficient ventilatory effects that they were measurable with the pressure sensor at the prothoracic spiracle (Fig. 14). This suggests that the splitting of aorta hemolymph flow in the neck serves for a more efficient hemolymph distribution, rather than for a damping of the aorta pulse.

5.6. The cellular arrangement, openings and valve structures of the dipteran heart

Most authors found only four heart chambers in higher flies, each with one pair of ostia (Lowne, 1893; Miller, 1950; Normann, 1972). In a more detailed light microscopic analysis, five incurrent ostia, and the construction of the heart tube of paired cells was described (Jensen, 1973). Jensen found 24 pairs in the larva, four of which were assumed to divide each into the two ostial cells on both sides with smaller nuclei during metamorphosis, representing the anterior four pairs of adult ostia, while the fifth (=posterior) pair of ostia is identical with the anterior larval ostia, which persists together with the anterior part of the larval 'ventricle'. Thus, he obtained 26 pairs of heart cells (excluding two posterior pairs of abdominal aortal cells). Due to the lack of a sketch with numbered cells, it was, however, impossible to fully match the cell numbers and arrangement of his analysis with the set of 27 pairs of heart cells in the present study (Fig. 3b), because Jensen (1973) did not mention how many cells persist exactly from the larval ventricle at the posterior adult heart. While all authors assumed a closed posterior heart end, caudal excurrent openings were postulated and then documented in connection with the first recordings of the heartbeat reversals in a fly (Wasserthal, 1982b). These openings have no valve structure. Thus an efficient outflow during backward pulses should be possible only when the negative pressure in the abdominal hemocoel is higher than that of the diastolic posterior heart.

The lateral left–right-alternating arrangement of paired heart and aortal cells does not seem to be restricted to higher flies, but to be the normal situation in insects. In flies, the cells are however especially conspicuous by the large nuclei bulging with the surrounding sarcoplasm into the heart lumen. The dorsal zig-zag cell borders between neighbouring heart cells have been described already in Odonata (Zawarzin, 1911) and the lepidopteran heart also consists of paired cells (Wasserthal and Wasserthal, 1980b). The generalized insect heart with an irregular orientation of more than two heart cells per cross-section, as depicted in some textbooks (Weber and Weidner, 1974; Seifert, 1995) is possibly a more exceptional constellation.

Some of the heart cells situated at the intersegmental folds are reported to metamorphose to vacuolized cells (Jensen, 1973). They correspond probably to the spongy heart cells in *Drosophila* (Miller, 1950) and to the cellular valve pads in *Ceromasia* larvae (Pantel, 1914). The ultrastructure of cell pair 17 at the intersegmental fold between abdominal segments 3 and 4, bulging most conspicuously into the heart lumen, shows, however, no vacuolar or spongy, but rather a homogenous cytoplasm. These cellular pads are suggested to act as cushion-like valve structures which might impede the reflux when these heart cells are contracted and the peristaltic wave is underway. They should do so in both directions but might be important especially at the end of the backward-pulse period to prevent re-entry of the hemolymph just expelled into the abdominal hemocoel by the diastolic heart. A similar but less pronounced cellular pad at the level of the intersegmental border between segments 4 and 5, might work in the same way. The protruding cytoplasm of these heart cells represents an extreme enlargement of the sarcoplasm surrounding all nuclei at the lumen face of the heart tube also in the other unspecialized heart cells.

5.7. Innervation

Synapsing and neurosecretory innervation of the anterior thoracic aorta and anterior heart was documented electromicroscopically and discussed in connection to the effects of neurosecretions upon heart activity, especially heartrate with inhibitory segmental nerve function and stimulating neurosecretory axons (Normann, 1972). With the complementing description of a similar innervation in the meso- and metathoracic aorta and in the central and posterior heart, it can be stated that the entire dorsal vessel offers the structural basis for being neuronally regulated; this is further supported because the muscle membrane and vertical muscle cells of the CPO are supplied by synapsing and neurosecretory axon terminals; thus the control of its intermittent pulse activity and the coordination of the pauses with the backward-pulse periods of the heart are probable. A neuronal—not merely mechanical myogenic—control of the coordinated periods during forward beating is also suggested by the independent pulse rate of the CPO.

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