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Active regulation of respiration and circulation in pupae of the codling moth (*Cydia pomonella*)

K. Sláma^{a,*}, L. Neven^{1,b}

^a Institute of Entomology, Czech Academy of Sciences, Drnovská 507, 16100 Prague 6, Czech Republic

^b USDA-ARS Yakima Agricultural Research Laboratory, 5230 Konnowac Pass Road, Wapato, WA 98951, USA

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Abstract

Regulation of autonomic physiological functions has been investigated by means of multisensor electronic methods, including electrocardiographic recording of heartbeat, strain-gauge recording of extracardiac hemocoelic pulsations (EHPs), anemometric recording of air passage through spiracles and respirographic recording of O₂ consumption and CO₂ output. Pupae of *Cydia* exhibit continuous respiration without remarkable bursts of CO₂. The dorsal vessel of these pupae exhibited regular heartbeat reversals characterized by shorter intervals of faster (forward oriented or anterograde) pulsations and longer intervals of slower (backward oriented or retrograde) peristaltic waves. The periodically repeated EHPs were present during the whole pupal interecdysial period. The internal physiological mechanisms regulating the cardiac (heartbeat) and extracardiac (EHP) pulsations were completely independent for most of the pupal instar. Simultaneous multisensor analysis revealed that the anterograde heartbeat of the dorsal vessel had similar but not identical frequency with EHPs. During advanced pharate adult development, frequency of cardiac and extracardiac pulsation periods profoundly increased until almost uninterrupted pulsation activity towards adult eclosion. At this time, the cardiac and extracardiac pulsations occasionally performed in concert, which enhanced considerably the efficacy of hemolymph circulation in pharate adults with high metabolic rates. The fastest hemolymph flow through the main body cavity was always associated with EHPs and with anterograde heartbeat. Simple physical diffusion of O₂ and CO₂ through spiracles (diffusion theory of insect respiration) does not play a significant role in pupal respiration. Instead, several kinds of regulated, mechanical ventilations of the tracheal system, including EHPs are responsible for effective tracheal ventilation. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

Interactions between hemolymph circulation and tracheal ventilation in large saturniid or sphingid pupae have been a common subject of discussions in the literature (see Wasserthal, 1996 for a review). The most spectacular physiological features that have been found in these giant pupae are the repeated switchovers between forward- and backward-oriented pulsations of the heart (see reviews by Jones, 1977; Wasserthal 1980, 1981; Miller 1985, 1997). As far as respiratory features are concerned, these giant pupae show large discontinuous

outbursts of respiratory CO₂ once in several hours (Buck, 1962; Brockway and Schneiderman, 1967; Miller, 1974; Kestler, 1985; Lighton, 1996; Wasserthal, 1996). A few reports related to insects of small body size, i.e. 500- to 10,000-fold smaller than saturniids, revealed the existence of special circulatory and respiratory adaptations that did not fit the general concepts. For example, miniature pupae of a leek moth (*Acrolepiopsis assectella*) actively regulated tracheal ventilation by means of special, rhythmically repeated extracardiac pulsations in hemocoelic pressure (Sláma, 1984b). Moreover, diapausing adults of a tiny bruchid beetle (*Bruchus affinis*, 5 mg body mass) did not show any stereotypic discontinuous ventilation cycles (DVC, see Lighton, 1996), which would be composed of three stages: open, fluttering and closed spiracle stages. Instead, they exhibited simple, highly abbreviated cycles

* Corresponding author. Tel./fax: +420-2-330-22482.

E-mail addresses: slama@entu.cas.cz (K. Sláma), neven@yarl.ars.usda.gov (L. Neven).

¹ Tel.: +509-454-6556; fax: +509-454-5646.

characterized by intermittent emissions of 30–40 nl of gaseous CO₂ in 3–5 min intervals. The concentrated CO₂ was expelled from the body by coordinated pumping movements of dorsoventral abdominal muscles (Sláma and Coquillaud, 1992; Sláma, 1999). The discovery of extracardiac hemocoelic pulsations (EHPs) in giant saturniid pupae (*Hyalophora cecropia*, Sláma, 1984b; *Attacus atlas*, Sláma, 1999) as well as in the pupal stages of other insect groups, led Sláma (1999) to conclude that perhaps all insects, whether large or small, could actively regulate their respiration. It was also suggested that this kind of active insect breathing could be mediated by a newly identified, autonomic, parasympathetic-like nervous system (coelopulse) with the centers located in the thoracic ganglia.

Recent development of new electronic methods (Sláma 1999, 2000) makes possible monitoring of the cardiac and extracardiac hemocoelic pulsations to record the respiratory exchange in insects smaller in size than 1 mm. In this study we have investigated regulation of some autonomic physiological functions in pupae of rather small, but economically important species of the codling moth, *Cydia pomonella*, in the hope that our findings could be perhaps used later in the integrated methods for controlling codling moth in fruit (Neven and Mitcham, 1996; Neven 1998a,b, 2000; Neven et al., 2001).

2. Material and methods

Freshly ecdysed pupae were collected daily from stock cultures and incubated within their cocoons in the dark at 25°C. Pupae were obtained from mass rearings of nondiapausing larvae fed common artificial diets at 23°C, 16:8 L/D photoperiod. The methods of recording changes in hemocoelic pressure, cardiac and extracardiac pulsations, respiratory movements, passage of air through the spiracles and O₂ consumption have been described earlier for pupae of other insect species by Sláma (1984a,b, 1988, 1999, 2000), Sláma and Denlinger (1992) and Sláma and Miller (2001). Heartbeat was measured using miniature thermographic sensors (oval shaped thermistors, 300×600 μm, type 10NR17 manufactured by PRAMET Co., Sumpark, Czech Rep., 120–270 Ω). The actively measuring thermistors were fixed by polyacetate glue on the rigid, second or third abdominal tergite (fourth abdominal segment can freely move). With the compensating thermistor twins they were connected in neighbouring branches of resistance bridges, with a constant, 5 kHz AC bridge feeding current (options of 1, 2 or 4 V). Modulated AC signals from the sensors were decoded, amplified and converted to DC output voltage by a 4-channel tensiometric unit (Mikrotechna, Prague, Czech Rep.) before being recorded on a PC or a battery of linear recorders.

Indirect recording of changes in hemocoelic pressure was realized by monitoring the movements of terminal abdominal segments by means of semiconductor strain gauges (Type AP-115-3-12, Podnik sluzeb, Zlin, Czech Rep.). The construction of strain-gauge transducers used in this work was previously described by Sláma (1984b). We also used the microanemometric method described by Sláma (1988), microrespirographic technique for recording O₂ consumption and CO₂ output in pupae by Sláma (1984a) and Sláma and Denlinger (1992), and a flow-through method for direct recording of pupal CO₂ output (Sláma, 1999). A method of simple visual observation and recording of heartbeat through transparent cuticle of abdominal tergites was described by Sláma (2000).

The results presented in the figures reveal selected examples representing usually a small fraction taken from a continuous, 4 to 24-h recordings. This evaluation of the data was selected because the main physiological accent of this study was to reveal relative differences between pulsations of different nature. Attempts to average pulsations with sinusoidal courses resulted in substantial loss of individual patterns. We also refrained from performing repetitive recordings required for statistical analysis. Electronic methods we used were extremely useful for recording pulsations or oscillations of all kinds but they were less suitable for determination of absolute values, which were often influenced by differences in positioning of the sensors. The results obtained on changes in pupal heartbeat were based on thermographic recordings in four individual pupae that were periodically measured during the whole pupal period. Posimetric data on extracardiac pulsations and heartbeat were based on continuous, 24 h-recordings from tip of the abdomen in 11 pupae of different ages. Anemometric recordings of air passage through spiracles on the second abdominal segment were realized in four specimens; direct recording of hemocoelic pressure in six specimens; recordings of CO₂ output by the flow-through method was made in three pupae; and, respirographic recordings of O₂ consumption and CO₂ output in five pupae of different ages. In addition to the above-listed single-sensor recordings, we recorded simultaneously multiple physiological functions using combination of several sensors in 14 individual pupae.

The data presented in the figures give physical measures that were derived from electrical values from calibration curves constructed specifically for each type of sensor. Thermographic results are expressed in °C, strain-gauge recordings in mm, changes in hemocoelic pressure in Pa and anemometric results in nl or μl of air movement. Oxygen consumption or CO₂ output are expressed in μl volumes. In order to cope with conventional organization of similar graphic charts, we illustrated abdominal contraction movement or the increased hemolymph flow in an upward direction.

3. Results

3.1. The nature of extracardiac hemocoelic pulsations

Morphologically, codling moth pupae differ from most other lepidopteran pupae by a complete absence of functional prothoracic spiracles and also by presence of a supernumerary, elastic intersegmental membrane between the 3A and 4A segments (unpublished observations). The presence of four instead of the usual three freely mobile abdominal segments enables the codling moth pupae a much wider abdominal rotation facilitating eclosion of the moth.

Perhaps the most conspicuous physiological feature of small lepidopteran pupae, such as the codling moth, is the presence of special EHPs in mechanical pressure of the hemocoelic body cavity. Duration and periodicity of these pulsations undergo constant variations during the course of pupal and adult development. The pattern of EHPs can thus be used as a convenient physiological criterion for determination of separate stages in pupal development. In young pupae (0 to 3-day-old), for instance, there are individual pulsations separated by relatively long periods of rest. These resting periods become successively shorter and the pulsations become more frequent during adult development in the second-half period, until there is almost uninterrupted pulsation activity towards adult eclosion. EHPs were present in these small pupae throughout the entire pupal interecydial period.

The common pattern of EHP during initial stages of pharate adult development can be observed in Fig. 1. This pattern of active EHP alternating in 5 min intervals

with relative periods of rest is characteristic for all pupae with pigmented eyes ($n=6$). The indirect recording of EHPs is a noninvasive physiological technique, which depends merely on gentle touching of the tip of the abdomen to a filament of the strain-gauge sensor (the load of the filament can be adjusted to less than 1 mg). The lower record in Fig. 1 shows more clearly that the individual extracardiac strokes of the abdominal pressure pump cause a prolongation, not a contraction (which is the most common case in all lepidopteran pupae of large body size, see Sláma, 1984b). This indicates that the contracting abdominal muscles involved in EHPs of the codling moth pupae include mostly the dorsoventral muscular groups, whereas the strong intersegmental abdominal musculature has been used here for other movements (see later).

3.2. Interactions between the cardiac and extracardiac pulsations (visual observations)

To avoid confusion between the cardiac and extracardiac pulsations, we have recorded EHP as described earlier and, simultaneously; have visually observed and recorded the systolic contractions of the dorsal vessel by hand (Fig. 2). Compared with the heartbeat, EHP were more uniform and regular. At 24°C they always started with somewhat higher initial frequency close to 1 Hz (57 strokes/min), whereas they ended usually with a frequency of some 0.75 Hz (45 strokes/min). There were no individual extracardiac strokes during the resting periods.

The heartbeat records in Fig. 2 (lower traces) revealed two different frequencies. The faster heartbeat was

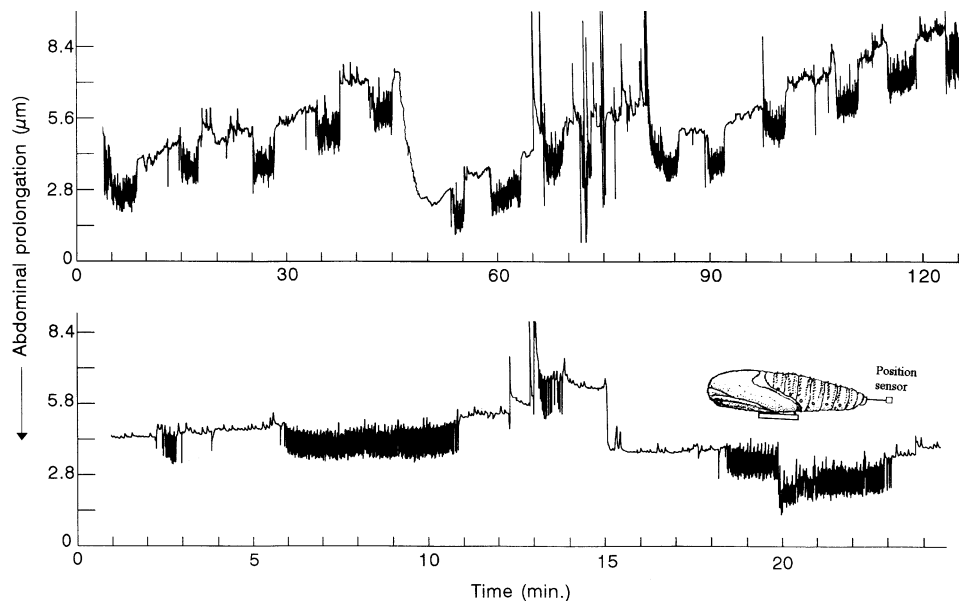


Fig. 1. Upper record shows a 2 h interval of EHPs revealed by means of position sensors attached to the tip of pupal abdomen. The lower record shows two regular series of EHP with slightly increased time scale. The pupa of codling moth was at the beginning of pharate adult development (pigmented eyes; 24°C).

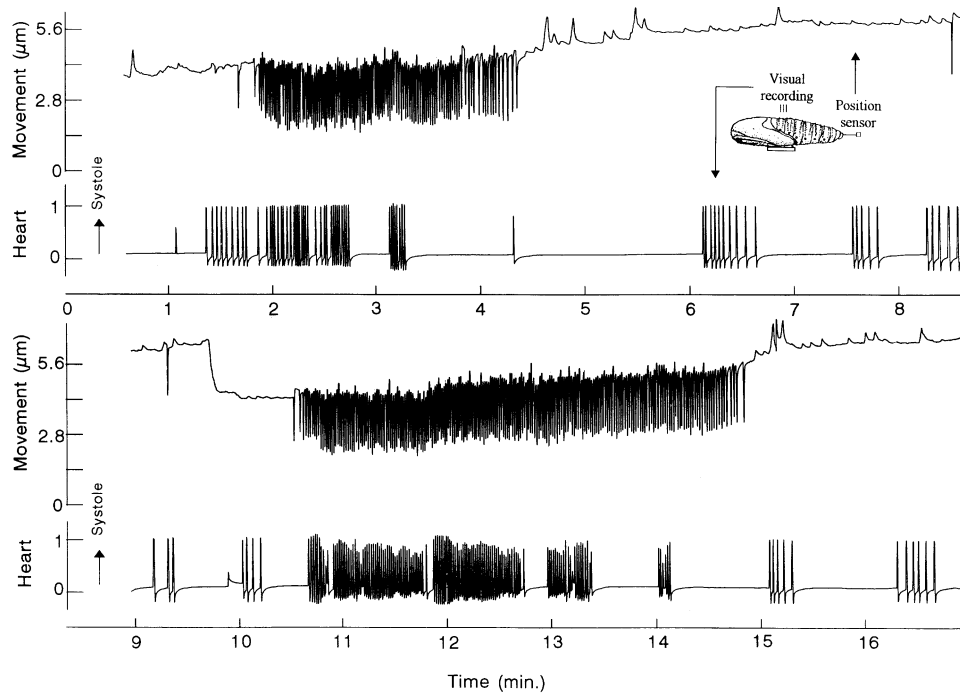


Fig. 2. A 16 min continuous interval showing the EHP as in Fig. 1 (upper traces) in combination with simultaneous recording of the pupal heartbeat. The systolic contractions of dorsal vessel (lower traces) were recorded by visual observation of the heart through transparent integument of the third abdominal tergite and manually pressing a switch at each peristaltic wave. The lower frequency of the backward oriented retrograde heartbeat can be found between minutes 6 and 10, while that of the anterograde heartbeat can be best observed between minutes 11 and 12 (24°C).

related to the forward oriented or anterograde peristaltic pulsations. The visual technique allowed in this case direct observation and unequivocal identification of the forward-oriented propagation of each peristaltic wave of the heart. It is very important to recognize in Fig. 2, that the velocity of anterograde heartbeat was almost identical with that of the EHP (57 systolic strokes/min). This clearly shows that, without simultaneous recording, anterograde heartbeat could be easily confused with the vigorous EHP. In addition, the possibility of confusion is further increased by the fact that both these physiologically different types of the hemocoelic pulsations are usually performed during similar intervals with similar, but not identical periodicity (see minutes 11–14 in Fig. 2).

Visual observations of the heartbeat in Fig. 2 revealed that the slow, retrograde systolic strokes of the heart indeed corresponded with the backward oriented peristaltic cardiac waves. The frequency of the retrograde heartbeat usually varied from 18 to 26 systolic strokes per minute (0.3–0.43 Hz). In contrast to the EHP, the retrograde heartbeat usually occurred in localized series of only a few pulses or just one large retrograde contraction during the relatively long periods of complete cardiac arrest. Similarities between anterograde cardiac and extracardiac pulsations may explain why the EHP remained undiscovered for a long time. In spite of the apparent similarities, however, we concluded that cardiac and extracardiac pulsations of these small lepidop-

teran pupae are regulated by two different physiological systems.

3.3. Recording of cardiac and extracardiac pulsations from the body surface

In addition to visual observation of insect heartbeat, discrete functions of the heart can be monitored by miniature thermistors situated on the integumental surface, just above the pericardial sinus. In pupae of the codling moth we have located the sensors in the middle of the first to third abdominal tergites. These segments are rigidly fused with anterior part of the body. They are most suitable for recording of anterograde heartbeat, while retrograde heartbeat may not be properly displayed because it only starts in the anterior part of the abdomen. The fourth, fifth and sixth abdominal segments are freely mobile and the sensors mounted on these segments showed interferences with EHP; artifacts due to large abdominal movements were produced.

Fig. 3 shows a small part of a prolonged thermographic recording of heartbeat in a relatively young pupa (2 to 3-day-old). The thermistor located on the third abdominal tergite (see Fig. 3 inset) records all subepidermal movements of the underlying hemolymph. An upward movement of the pen driver is directly proportional to the increased flow of hemolymph through the perineural sinus. Conversely, the baseline at the beginning and at the end of Fig. 3 indicates a minimal

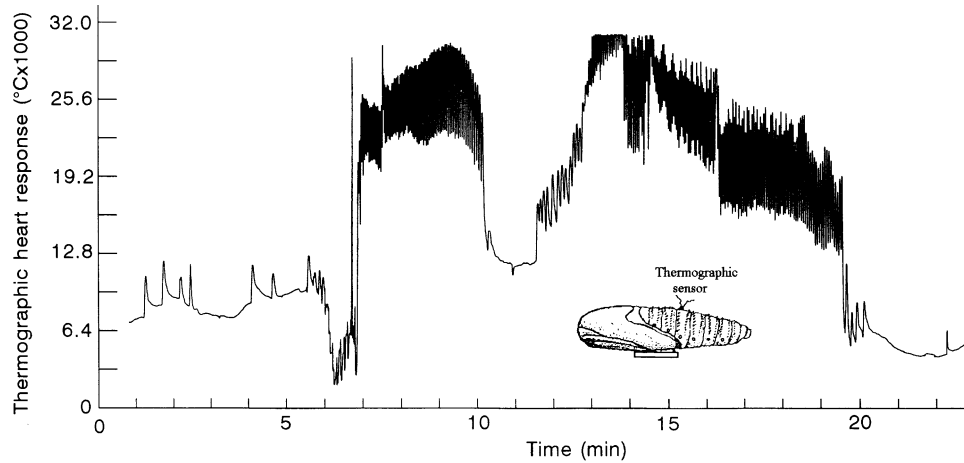


Fig. 3. Thermographic record realized through thermistor located on the third abdominal tergite of relatively young pupa with still unpigmented eyes. Note that an increased hemolymph flow under the thermistor is proportional to the upward movement of the pen driver. The two bouts of anterograde heartbeat (7–10 and 13–20 min.) have been associated with the most intensive hemolymph flow.

hemolymph flow during the period of relative cardiac arrest. There were two distinctive bouts of the faster anterograde heartbeat in Fig. 3, which are connected with the most intensive hemolymph flow. They are terminated by complete cardiac rest (10–11 min) or, eventually, by a few retrograde strokes before the rest (close to minute 20). The retrograde heartbeat can be found in Fig. 3 also at the beginning of each bout of the anterograde activity (cf. minutes 5–7 and 12–13). The reversal of hemolymph flow from retrograde to anterograde direction was immediate. The isolated, individual retrograde systolic contractions of the heart (see minute 0–5) have relatively smaller effects on hemolymph flow. In addition, the twin bouts of anterograde pulsations with relatively long periods of rest, as shown in Fig. 3, were characteristic for the heartbeat in young pupae (first third of the pupal period). Occasionally, the bouts were separated by one or several hours of complete cardiac as well as extracardiac arrest.

The most important physiological relationships were found by simultaneous recordings of the cardiac and extracardiac pulsations. Some results of the combined thermographic and strain-gauge measurements are shown in Fig. 4. There are four distinctive bouts of anterograde cardiac as well as extracardiac pulsations, which are separated by 5–10 min periods of relative arrest. Note in Fig. 4 that the bouts of anterograde heartbeat (traces A) always preceded initiation of the EHP (traces B). From this one might logically deduce that the periods of these pulsations might be actually determined by periodicity of the anterograde heartbeat. Unfortunately, this assumption cannot be supported by other measurements. Extensive recordings of this kind with other pupae revealed that the relationships shown in Fig. 4 might be common, but not causally related. This conclusion was supported by the fact that numerous other records showed, alternatively, that EHP could start the

mutual bouts of pulsations or, eventually, both types of these pulsations alternated in time. Therefore, there was no coordination between the cardiac and extracardiac pulsations (except for the advanced pharate adult period a few days before adult eclosion).

In addition to the pulsations described above, Fig. 4 shows that there are sudden expiratory abdominal contractions (individual upward peaks on B traces, see later), or inspiratory abdominal elongations (downward peaks on traces B) or, occasionally, large abdominal rotations, occurring at 30 min intervals (indicated on traces B as large up and down movements). These movements have respiratory rather than circulatory functions, as noted later. The most intensive hemolymph flow (upward position in A) has always been correlated with the terminal part of the EHP.

3.4. Further temporal relationships between cardiac and extracardiac pulsations

The simultaneous record of hemocoelic pulsations in Fig. 5 shows quite different temporal relationships in comparison with the data presented in Fig. 4. Bouts of anterograde heartbeat did not correlate, as can be seen in Fig. 5, but alternated with the bouts of EHP. The thermographic portion of these records (upper trace) represents in fact a very nice example of the heartbeat reversal in a lepidopteran pupa of small size. Note the isolated series of low-frequency, retrograde heartbeat (see 0–3 min and 4.6–8 min), which are interrupted by much faster, forward oriented anterograde heartbeat (indicated by A line).

The results presented in Fig. 5 demonstrate that regular heartbeat reversal, so far known mostly from the giant saturniid or sphingid pupae, may constitute physiological mechanism that is essential for sufficient circulation of hemolymph in pupae of small body size. More-

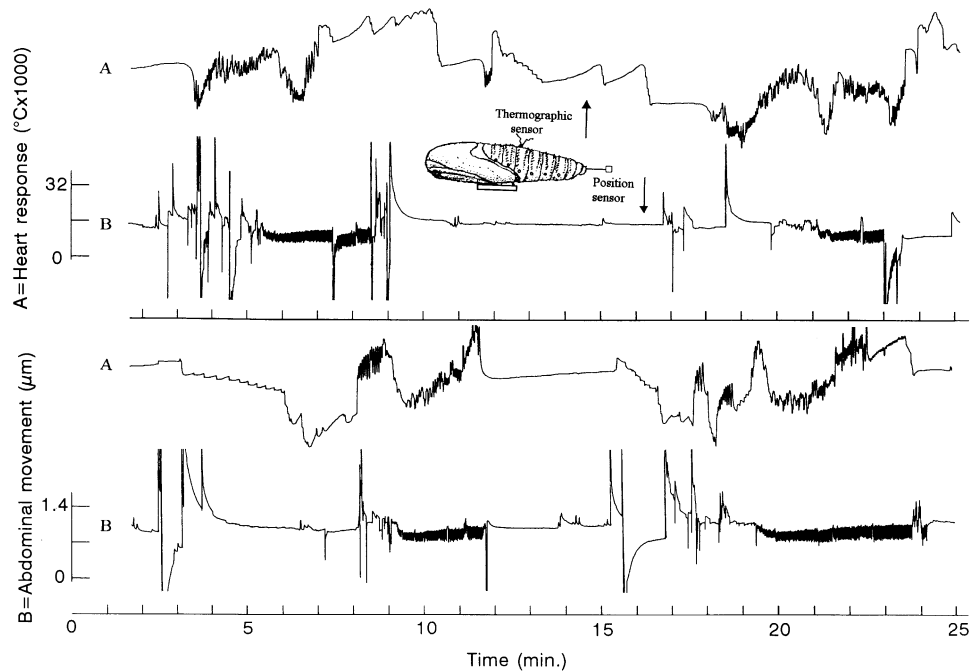


Fig. 4. Simultaneous, thermographic–positometric recording, which shows the interplay of the anterograde cardiac (trace A) and the extracardiac (trace B) pulsations over the period of 2×25 min. Note that the two kinds of physiologically different, A and B pulsations, occur with similar periodicity, at similar intervals of time and also with quite similar, but not identical frequency. The purely abdominal, retrograde heartbeat has not been well expressed in this case due to placement of the sensor near front end of the abdomen (first abdominal tergite). The pupa used in this record was in about the middle of the pupal instar, with slightly pigmented eyes, at 25°C.

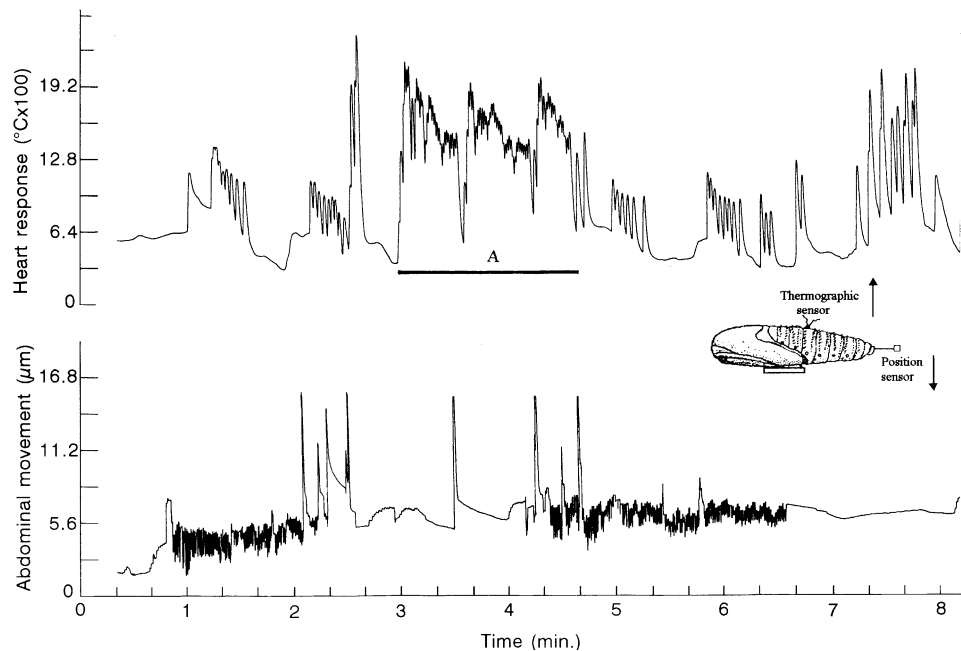


Fig. 5. Results of the combined thermographic–positometric recording, which shows characteristic example of heartbeat reversal (upper record, line A indicates anterograde heartbeat) in codling moth pupa occurring in the middle of pupal instar; at room temperature. The bottom record shows simultaneously two EHP and eight expiratory abdominal movements (sudden peaks of abdominal contraction).

over, we can also observe that the beginning and switchover between the two reciprocal peristaltic waves of the heart is instantaneous. This is also true for the switchover between anterograde and retrograde heart-

beats (see 4–7 min). This involves in the giant pupae as a rule a more or less extended period of temporary cardiac rest.

The bottom record in Fig. 5 shows two EHP before

and after a bout of anterograde heartbeat. This example of alternating pulsations has been purposely selected from the prolonged recording in which the bouts of anterograde heartbeat had somewhat longer periodicity than EHP. We could thus observe continuous phase shifts between the two kinds of hemocoelic pulsations, suggesting existence of two, absolutely independent, regulatory mechanisms. This conclusion can be further supported by the occurrence of pseudo-synchronization, when two similar chronological events occasionally merge together for some time and then separate again. According to this, for example, the anterograde heartbeat can often be found running parallel with the EHP, but sooner or later the cardiac and extracardiac pulsations deviate and begin to run asynchronously. Several such exchanges of the synchronous and asynchronous cardiac–extracardiac patterns have been observed during the uninterrupted, 24 h recordings in four specimens (age in the middle of pupal period).

While the cardiac and extracardiac types of hemocoelic pulsations appear to be driven by different and mutually independent physiological mechanisms, similarities in the periodicity and duration of the pulsations occur. In addition, there are similarities in the decreasing frequency of the anterograde heartbeat and EHP. This suggests that separate cardiac and extracardiac regulatory systems might be under mutual control of some common homeostatic, feedback mechanism, which is moderating all kinds of endogenous excess or deficiency among hemolymph and tissues.

The conclusions about independent regulation of the cardiac and extracardiac pulsations are valid for the major part of the pupal interecdysial period with exception of the advanced pharate adult stage approaching adult eclosion (pigmented wings and abdomen). At this terminal period of enormously elevated metabolism, both cardiac and extracardiac systems occur in the state of continuous, uninterrupted pulsation activity. There are certain indications that both of these different cardiac and extracardiac systems act in concert at the terminal period, which would substantially enhance the rate of hemolymph circulation throughout the body.

During the last two days before adult eclosion, the resting periods between all types of hemocoelic pulsations successively diminish. Vigorous EHP appear to be correlated with the terminal part of the anterograde heartbeat or, with a few retrograde strokes that follow immediately afterwards. This situation is documented in Fig. 6, which shows the effect of EHP on thermographic record of the heartbeat. The upper record shows a typical example of the merging of two different frequencies during the anterograde pulsation. During evaluation of the records in Fig. 6 we have obtained an impression that the retrograde pulses became synchronized with every second extracardiac stroke at the end of the pulsation (close to minute 5). The frequency of the retrograde

heartbeat shows at this time exactly half of the frequency of EHP (24/min to 48/min, or 0.4–0.8 Hz, respectively) and this ‘synchronized’ frequency is smaller in comparison with the first bout of retrograde pulsation realized before initiation of the EHP (30/min, or 0.5 Hz, see minute 2 in Fig. 6).

3.5. *The circulatory and respiratory roles of large abdominal contractions*

In addition to EHP, which are usually produced by abdominal movements as small as 1 μm , we can observe two additional types of large abdominal movements with 50- to 250-fold larger amplitude. These abdominal movements are present in pupae of all ages. They are repeated in regular intervals, the frequency of their occurrence increases enormously at the end of the pupal period. This shows that they are essential for successful pupal development and eclosion. The first type is represented by instantaneous, convulsion-like abdominal contractions along the longitudinal axis. The second type of these movements appears as single or multiple abdominal rotations (Fig. 7).

In Fig. 7 we can observe one six-fold and one five-fold abdominal rotation of close to minute 1. Another such abdominal rotation occurred closely after minute 4. It is essential to realize that the large up and down oscillations of internal hemocoelic pressure associated with these multiple abdominal rotations would inevitably produce an outflow or inflow of gas from any opened spiracle. The first type of the above-mentioned abdominal movements, the brief, convulsion-like abdominal contractions, appear on lower record in Fig. 7 as large positive peaks. These movements are used for active ventilation of large proximal part of the pupal body, mainly through the spiracles located on the second abdominal segment (for more details see the following sections).

Comparison of the simultaneous posimetric (Fig. 7, later) and thermographic (earlier) records can be used for demonstration of non-specificity of the thermographic method for recording only the heartbeat. It clearly shows that abdominal contractions can produce a subcutaneous movement of hemolymph under the sensor located outside of pericardial region of the rigid third abdominal tergite, thus giving a false impression of functions of the heart. Conversely, it can be noticed in Fig. 7 (lower record) that the impact of true anterograde heartbeat on pupal abdominal movement is extremely small. As we can see, the heartbeat could hardly be discerned among the large abdominal movements. It can be mentioned, finally, that the upper thermographic record in Fig. 7 also provides a beautiful example for successively decreasing frequency in the course of one complete anterograde pulsation of the heart.

The large abdominal movements described in Fig. 7

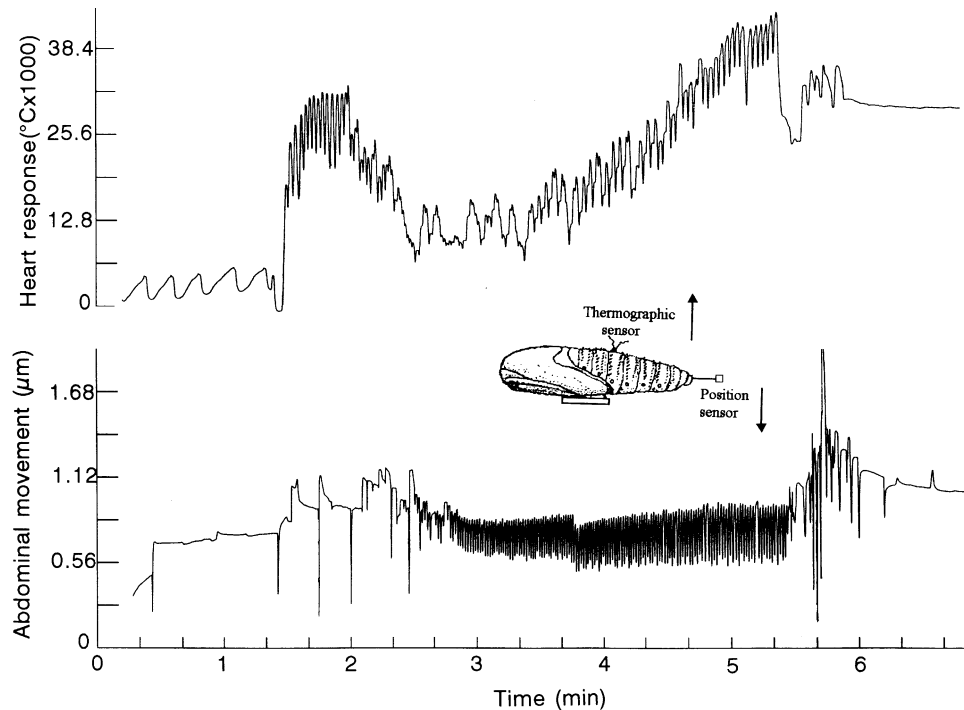


Fig. 6. Results of simultaneous thermographic–posimetric recording showing retrograde (minutes 0–2.3) and antero-gradate (minutes 2.3–5.3) cardiac pulsations during one regular EHP (bottom record). The investigated pupa showed signs of advanced adult development. The vigorous extracardiac pulsation produces irregularities in the heartbeat, which is quite common feature on the thermographic records. By contrast, heartbeat cannot influence the strain-gauge pattern of EHP, because it gives about 50-fold weaker responses at the tip of the abdomen.

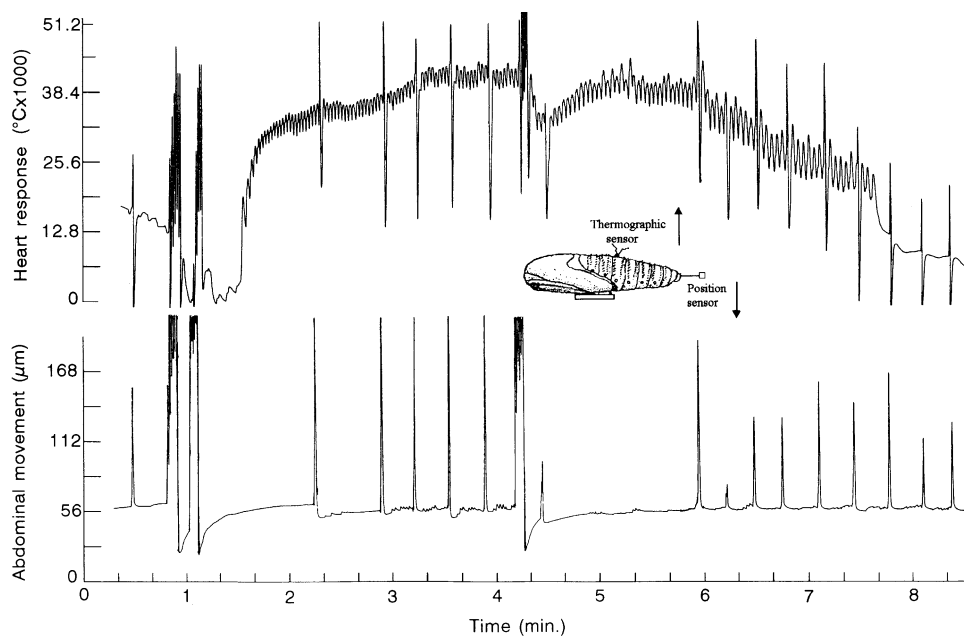


Fig. 7. Results of the combined, high resolution, thermographic–posimetric recordings during one complete bout of antero-gradate heartbeat (see upper record) in younger pupa with still unpigmented eyes. Lower, simultaneous record shows two types of large abdominal movements recorded by posimetric sensors from the tip of abdomen during the resting period between EHP. The first type has been made by sudden abdominal contractions (amplitude 50–100 μm , see minutes 2–4), the second type corresponds to multiple abdominal rotations (close to minutes 1 and 4). Note that the thermographic sensor on third abdominal tergite has also recorded all these large abdominal movements (cf. upper and lower records), giving false impression of heart function.

should obviously have a large impact on tracheal ventilation. From these data we can calculate that, for example, an abdominal contraction of average size of 110 μm (usual range is from 50 to 160 μm) should cause a 0.2 μl change of internal volume (based on 1.766 mm^2 cross-section through the median flexible abdominal segment). This shows that, with three such peaks per minute as shown in Fig. 7, these movements alone would yield the ventilatory capacity equal to 0.6 μl of air per minute, which is quite a large value considering relatively small intratracheal space of the pupa. In addition, it can be expected from the data contained on lower record in Fig. 7 that the ventilatory impact of vigorous abdominal rotations should be correspondingly much larger, while a possible ventilatory effect of the anterograde heartbeat should be negligible.

3.6. The direct recording of hemocoelic pressure

The direct recording of changes in hemocoelic pressure represents a major challenge for the small pupae of the codling moth. The insertion of hydraulic transducers into the hemocoelic cavity requires the use of very thin hypodermic needles. In addition, the pupal hemolymph has strong coagulating capacity, which often results in occlusion of the measuring needle. Nevertheless, we have obtained 11 successful records that can be used for essential physiological calculations. These records, such as the one in Fig. 8, provide the first results of direct measurements of hemocoelic pressure in very small insects.

We have found that young codling moth pupae with still unpigmented eyes maintain the atmospheric zero, or slightly subatmospheric, negative baseline hemocoelic pressures reaching usually upto -200 Pa ($n=6$). By contrast, the developing pharate adults with pigmented adult eyes (second half of pupal period) always show positive, i.e. higher than atmospheric hemocoelic pressures ranging from 0 to $+250$ Pa ($n=5$). The advanced pharate

adults less than two days before adult ecdysis show highly positive hemocoelic pressures of up to $+0.5$ kPa or more before rupturing the old pupal cuticle.

In Fig. 8 we show a fragment of one direct hemocoelic pressure record obtained with a pupa during pharate adult development. The record shows the impact of sudden abdominal contractions (Fig. 7) on hemocoelic pressure. By comparing the data in Figs. 7 and 8 we can find that the brief abdominal contractions of 110 μm average size produce positive peaks of 200 to 500 Pa in hemocoelic pressure. This indicates that the conversion factor between abdominal contractions and hemocoelic pressure would be close to 1 μm of abdominal movement producing a 3–4 Pa change in pressure. Based on this conversion factor we can estimate that extracardiac pulsations of an average amplitude of 1 μm would produce rather small oscillations in hemocoelic pressure of only 3–4 Pa ($=0.3$ – 0.4 mm of hydrostatic pressure).

3.7. Passive suction inspirations in young codling moth pupae

The symptoms of intermittent, passive suction inspirations (PSIs), become visible only when a pupa is able to tightly constrict all spiracular valves. When this happens, hemocoelic pressure steadily decreases with the velocity corresponding to O_2 consumption, while the metabolic CO_2 is dissolved in bicarbonate buffers. When a spiracular valve opens, air flushes inside the tracheal system and hemocoelic pressure shows a temporary rise. The described ‘saw tooth pattern’ in hemocoelic pressure can be conveniently monitored from outside of the body by recording the corresponding, passive retraction of the terminal abdominal segments.

The pupae of codling moth exhibit the PSI pattern only in the young pupal stages (still unpigmented eyes) characterized by relatively low metabolic rates (O_2 consumption between 200–400 $\mu\text{l/g/h}$). In a given specimen the characteristic patterns of PSI alternate with the per-

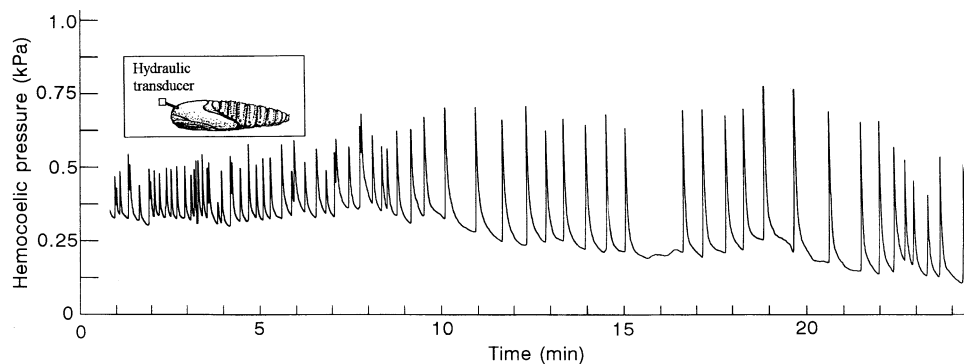


Fig. 8. Result of direct recording of changes in hemocoelic pressure in the developing pharate adult stage, one or two days before adult eclosion. The hydraulic transducer of mechanical pressure (31 gauge stainless steel needle) was inserted into the body cavity through mesonotum. Note that the baseline pressure is higher in comparison with atmospheric zero pressure; the peaks (150–500 Pa) have been created by sudden abdominal contractions.

iods without the PSI, which indicates that one or more spiracles remain incompletely constricted for longer intervals of time. Fig. 9 shows the PSI pattern in a young pupa during the resting period after termination of the cardiac and extracardiac pulsations (15–24 min). This pupa made nine inspirations of air ('teeth') per minute. In contrast to young pupae with still unpigmented eyes, the developing pharate adults show several kinds of very frequent hemocoelic pulsations, but are unable to perform the characteristic PSI pattern. This provides indirect evidence to show that these stages with considerably increased metabolic rates (800–1200 μl of $\text{O}_2/\text{g}/\text{h}$) are unable to close all spiracles of the body for longer intervals of time.

During these measurements we have found that monitoring of PSI patterns could be effectively used for obtaining physiological information concerning the rate of respiratory metabolism, functioning of spiracles, and regularity of inspirations. The practical utility of PSI monitoring can be exemplified by a sample of abdominal movement record in Fig. 10. It reveals that young pupa with unpigmented eyes had a regular respiratory pattern with approximately nine inspirations per minute, with the velocity of constant, O_2 consumption dependent abdominal retraction of 11.76 μm per minute (indicated by the dotted line B in Fig. 10). We already know from the above-described data (Fig. 7) that a 110 μm of abdominal movement corresponds to 0.2 μl of internal volume change (i.e. 1 μm movement = 1.8 nl internal V). From this we can easily calculate that the constant velocity of 11.76 μm of abdominal contraction per minute

(see B in Fig. 10) should correspond to a constant O_2 consumption of 21.16 nl/min or 1.27 $\mu\text{l}/\text{h}$. Further, high resolution recordings of this PSI pattern (not shown here) indicated that the interval of the separate spiracular valve openings during PSI lasted only 800 ms or less. With nine inspirations per minute, the spiracular valves opened only for 7.2 s/min, e.g. only for some 12% of total time. During the remaining 88% of time the pupal body was hermetically sealed from the outside air. This paradoxical situation explains possible reasons for the necessity of EHP, sudden abdominal contractions and abdominal rotations for sustained tracheal ventilation.

3.8. The effect of actively regulated abdominal movements on tracheal ventilation

In spite of recent availability of very sensitive microanemometric methods (Sláma, 1999), we were so far unable to realize recordings through individual spiracles. These pupae are too small. Their tracheal system is quite special because it does not include functional prothoracic spiracles. In addition, the spiracles of the first abdominal segment are inverted inside the body, being opened into a cavity under the wing lobes. Due to these features, the first pair of the proximally located functional spiracles are the ones located on the second abdominal segment. We have thus assumed that regulation of 2A spiracles would be crucial for sufficient ventilation of the large proximal part of the body, including head, thorax and the beginning of the abdomen. To avoid the above-indicated handicap in connecting single spir-

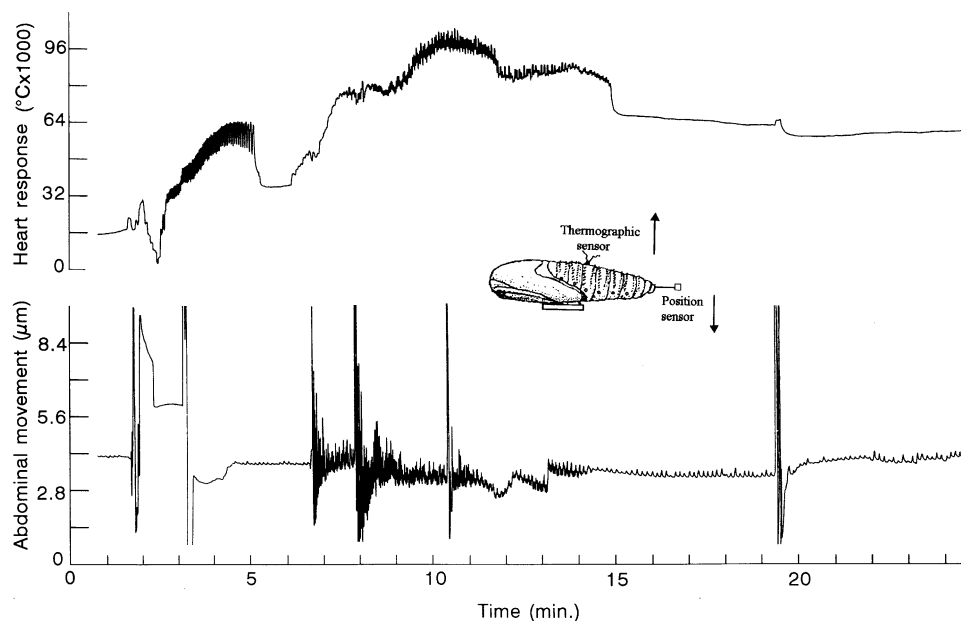


Fig. 9. The result of simultaneous thermographic-positometric recording with relatively young pupa (unpigmented eyes), which shows synchronization, or initiation of EHP between the two bouts of anterograde heartbeat. The pupa performed multiple abdominal rotations close to minutes 2, 3 and 20. During the period of cardiac as well as extracardiac rest (after minute 15) we can observe development of passive suction inspirations characterized by the common 'saw-tooth pattern' on the lower record.

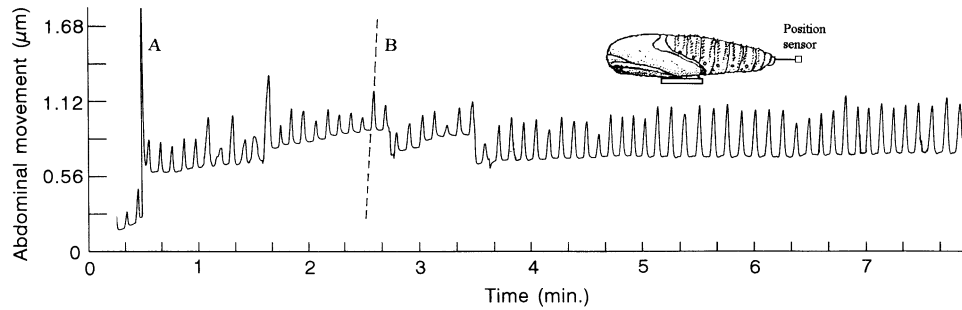


Fig. 10. Result of high-resolution recording of abdominal movements in a younger pupa exhibiting the passive suction inspirations. There are nine inspirations per minute characterized by instant prolongations of the abdomen (pen driver writes downwards) caused by sudden inflow of air through some spiracle. (A) indicates the effect of sudden abdominal contraction and (B) shows the velocity of constant abdominal contraction ($11.76 \mu\text{m}/\text{min}$, nine spiracular openings per minute, duration of spiracular opening 800 ms, and -200 to -400 Pa subatmospheric baseline hemocoelic pressure).

acles, we created two anemometric body compartments, which are shown by inset in Fig. 11. The A compartment contained the isolated pair of the important second abdominal spiracles, whereas the B compartment contained the remaining five pairs of the posterior spiracles.

The results of anemometric recording in Fig. 11 reveal at the first glance that all types of abdominal movements that have been described in the previous section can exert pronounced effects on exchange of respiratory gases between the second abdominal spiracles and the rest of the body. To understand these data, it is essential to know that anemometric sensors of Fig. 11 recorded specifically the inflow of air into spiracles in the upright direction, while the outflow of air was recorded downwards from the baseline. According to this, extracardiac

pulsations produce inflow of air into the second abdominal spiracles, which is associated with a 'mirror image', or reciprocal outflow of air from posterior abdominal spiracles in the B compartment (see minutes 6, 10, and 22). The same is true with respect to sudden abdominal contractions (between minutes 5 and 10 in Fig. 11, see also Fig. 7 for comparison), which are mostly associated with a rapid intake of fresh air in A and expulsion of intratracheal gas from some posterior spiracles in B. For practical reasons we call these sudden abdominal contractions the 'expiratory movements', although they actually represent combined out-and-in flows at the level of different body segments. This statement can be documented in Fig. 11 by reciprocal airflow associated with five abdominal movements between the 5th and 10th

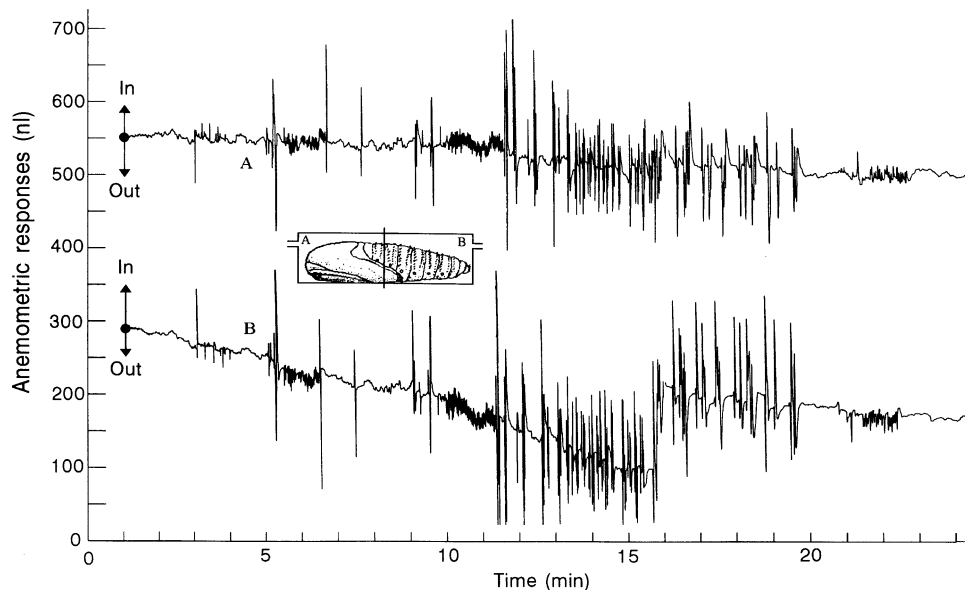


Fig. 11. Result of simultaneous microanemometric recording of the airflow between isolated anterior body compartment (A, functional spiracles of the second abdominal segment only), and the posterior body compartment (B, containing functional spiracles of the third to seventh segment). In some instances the records show a reciprocal mirror image pattern and selective filtration of air stream during EHP. The recording has been realized with a pupa — advanced pharate adult — with the elevated rate of respiration. Note that the movement of gas into the body (inspiration) was recorded upwards, expiration downwards from the baseline.

minute of the recording time. It shows that the nervous system of the pupa can convert the polarity of airflow through the spiracles.

The results presented in Fig. 11 offer a new look at insect breathing. They especially show two important things. The first conclusion is that a small pupa with micrometer distances needed for the diffusion of oxygen (best suited to fit the axioms of diffusional theory of insect respiration) avoids diffusion and uses mechanical ventilation as the main principle of the gas exchange. Moreover, ability of the pupa to control selectively the in- and out-flow of gas through second abdominal spiracles demonstrates that the pupae can actively regulate their breathing by means of a sophisticated neuromuscular system.

Fig. 12 shows a few more details related to the above-mentioned neuromuscular regulatory system. Here we can see a perfectly filtered, unidirectional stream of air into the second abdominal spiracles during each EHP (see 1–3, 6–7 and 9–10 min), which has been associated with a countercurrent reciprocal outflow of air from some of the posterior spiracles. The simple fact that this phenomenon could occur at all during the extracardiac pulsation has some important physiological implications. To achieve selective filtration of air stream as shown in

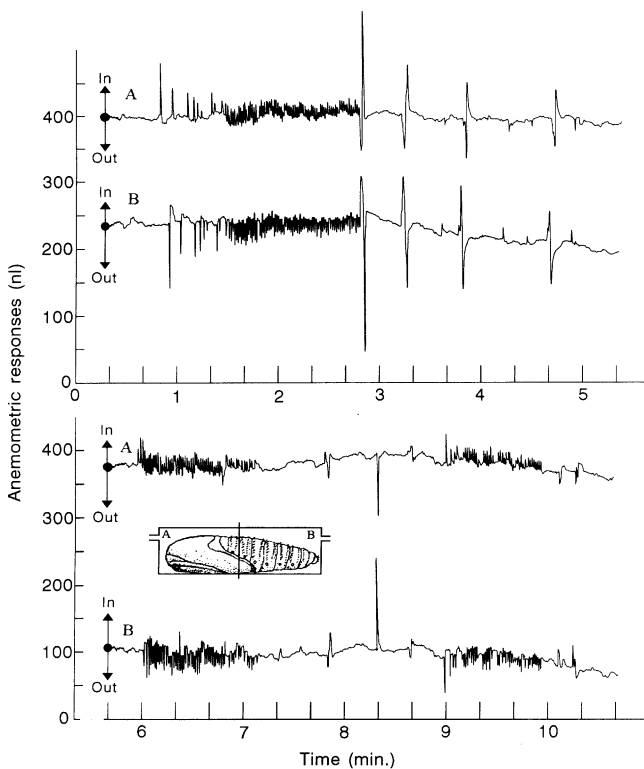


Fig. 12. Similar records as in Fig. 11, showing three EHP and several abdominal contractions in more detail. The extracardiac pulsations do not show tidal ventilation. They show selective filtration of air stream into the second abdominal spiracles. However, the pupa could also suddenly change the polarity of air stream in opposite direction (see minutes 8–9).

Fig. 12, the spiracular valves should open and close with the equivalent or higher frequency in comparison with that of the EHP. As the frequency of EHP is usually close to 1 Hz, this means that spiracular valves must also selectively pulsate with the velocity of at least 1 Hz. Perhaps the most surprising recognition that comes out of these results is that, to achieve the unidirectional and occasionally reciprocal stream, the nerve system that operates the spiracular valves has to be precisely synchronized with the respective upgoing or downgoing half-waves of the extracardiac pulse. This is also true for reciprocal changes in the polarity of sudden inspirations of air (see reciprocal airstreams at minutes 3 and 8).

The ventilatory capacity that is associated with sudden abdominal contractions in Fig. 12 is relatively large. The sudden intake of gas into the second abdominal spiracles at minute 3 reveals a bulk flow of 200 nl of air. With three such inspirations per minute the pupal tracheal system can receive 0.6 μ l of air per minute, which amounts to 36 μ l of air per hour. In addition, Fig. 12 also shows that each stroke of EHP would cause some 25 nl inflow of air, which corresponds at the frequency of 1 Hz to 1.5 μ l of mechanically inspired air per minute. With the average of 30% time that the pupae spend in EHP, this shows that the pupa inspires 30 μ l of fresh air only by EHP each hour. With respect to small pupal size, we conclude that sudden abdominal contractions, EHP and multiple abdominal rotations are sufficient for replacement of the whole intratracheal volume several times per hour.

3.9. Direct recording of changes in pupal respiratory metabolism

To obtain further informations about the mechanism of gas exchange in pupae of the codling moth, we have precisely measured the rate of their O_2 consumption and CO_2 output by means of the scanning respirometry method. The pupae of the codling moth are immobile and develop without diapause. We found that respirometric records of O_2 consumption always revealed a constant rates with a minimum at the second day after pupal ecdysis. Since this time, the pupal O_2 consumption rate regularly increased until its culmination during adult emergence. We have not encountered any larger bursts in CO_2 production, nor any small but frequent discharges of CO_2 in form of the Babak's respiratory cycles ($n=9$; Section 4). Fig. 13 illustrates the absolutely regular course of O_2 consumption obtained by the scanning microrespirometric record obtained with a pupa at the pharate adult stage (dark pigmented eyes, about three quarters of pupal period). We can see that the pupa regularly consumed oxygen at the rate of 480 nl per minute ($\approx 28.8 \mu$ l O_2 /h, or 823 μ l O_2 /g/h). The continuous and perfectly regular release of metabolic CO_2 from the body (determined by recording the ratio of CO_2 – O_2 in absence

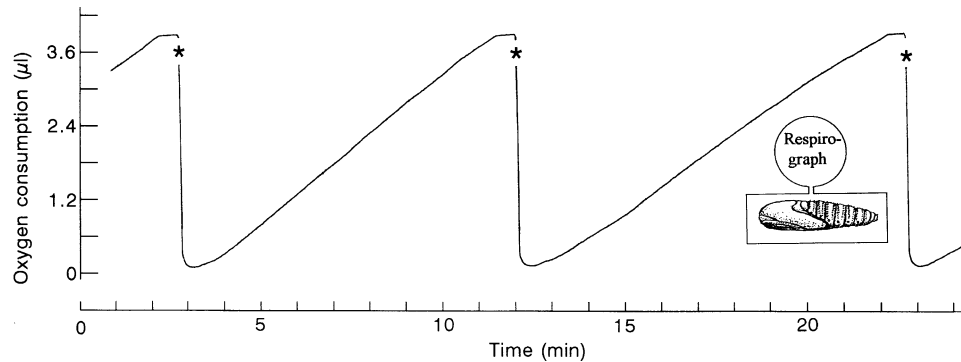


Fig. 13. A sample taken from the recording of pupal respiratory rate by the scanning microrespirographic method during the pharate adult development in a pupa of 35 mg body mass. The record shows a regular rate of O_2 consumption with the velocity of 480 nl of O_2 /min (=823 μ l of O_2 /g/h). Asterisks indicate the moments of electronic zeroing; 1% KOH was used for CO_2 absorption.

of CO_2 absorbent) demonstrates clearly that the metabolic formation of carbonic acid has been continuously neutralized by diffusion of gaseous CO_2 into the air-filled tracheal spaces. This type of continuous neutralization of the respiratory acidemia in insects (absence of CO_2 bursts) is more common than the discontinuous respiration found in the giant lepidopteran pupae. It is obvious that a continuous and very regular elimination of the metabolic CO_2 from the body requires assistance of extracardiac pulsations and other abdominal movements described in the previous sections.

4. Discussion

Since the 'classical' experiments of the late H.A. Schneiderman and his co-workers in the 1960s on discontinuous respiration in diapausing pupae of cecropia silkworm (Brockway and Schneiderman, 1967), relatively small experimental work has been done with respect to regulation of pupal respiration and hemolymph circulation. The data obtained on the giant pupae of cecropia were sophisticated and discussed in various review articles and textbooks, becoming thus a general axiom for all insects (Buck, 1962; Miller, 1974; Kestler, 1985; Sláma, 1988; Lighton, 1996; Wasserthal, 1996). We have found, however, that the relatively small pupae of the codling moth (some 250-fold smaller than *cecropia*) do not respect the common theories of DVC. By contrast, the regulation of autonomic physiological functions proceeds here in the form of absolutely acyclic, continuously regular O_2 consumption and CO_2 output rates (Fig. 13). With respect to internal physiological regulation, this indicates that the respiratory gases continuously diffuse from hemolymph and tissues into the air filled tracheal spaces. Then, the common problem of all insects and perhaps of all terrestrial arthropods is how to get sufficient amounts of O_2 in and CO_2 out of the body without the concomitant loss of essential amounts of H_2O vapor. The mechanistic theory

of insect respiration (diffusion theory of A. Krogh), which is contained in all textbooks of insect physiology, solved this problem on the basis of simple, passive physical gaseous diffusion through the spiracles. A more recent alternative to this problem claims that insects are able to ventilate the tracheal system actively by using the quite sophisticated, autonomic, cholinergic nervous system (coelopulse) which is associated with an 'abdominal pressure pump' (Sláma 1988, 1999). The results obtained in this work support the second alternative. They clearly show that the small pupae of *Cydia* avoid extensive diffusional water loss (Kestler, 1985) by minimal openings of the spiracular valves (Hazelhoff, 1927), which is supported by considerably more economic, actively regulated, mechanical ventilation of the body.

We have also found that the above-indicated 'abdominal pressure pump' works by means of special EHPs, occurring in regular intervals during the whole pupal–adult interecdysial period. As similar EHPs occur in pupae of all major groups of endopterygote insects (Sláma, 1999), they apparently play an important role in the maintenance of homeostasis and in regulation of the autonomic physiological functions in general. In this paper we have established that the actively regulated mechanical ventilations, such as are EHP, sudden abdominal contractions and occasional abdominal rotations, are more essential for ventilation of the tracheal system than diffusion (Figs. 11 and 12). This recognition is not quite new, neither is it limited only to insects of the small body size. Actually, the presence of EHP has been well documented earlier also in the giant pupae of *H. cecropia* (Sláma, 1984b) and *A. atlas* (Sláma, 1999). We can thus conclude that EHP and other abdominal movements play an important role in regulation of pupal respiration and hemolymph circulation in other insect species as well. Unfortunately, the phenomenon of EHP has been overlooked for a long time due to complexity and absence of adequate electronic techniques.

Kuusik and his co-workers (1992) analysed respiratory movements and spiracular flow of air in the pupae of *Galleria mellonella*. They described expiratory and inspiratory strokes of air through the spiracles that were associated with large abdominal movements. Later, Kuusik et al. (1996) reported about the existence of four types of external gas exchange in *Galleria*: (i) rapid release of CO₂ in intervals of 8–14 s; (ii) slow release of CO₂; (iii) chaotic rhythm of gas exchange; and (iv) continuous respiration. Our results obtained in *Cydia* are consistent only with the types (ii) and (iv), i.e. slow release of CO₂ and continuous respiration. Unlike in the pupae of *Galleria*, there were no rapid bursts of CO₂, nor any ‘chaotic’ rhythms of gas exchange in *Cydia*. Our results, demonstrating clearly active involvement of EHP in spiracular ventilation (Figs. 10 and 11), are in direct conflict with the conclusion of Kuusik et al. (1996) that: “externally imperceptible abdominal movements due to extracardiac pulsations of hemolymphal pressure played an inessential role in active tracheal ventilation”.

The described results concerning relationships between the cardiac and extracardiac pulsations in *Cydia* appear to be very similar as in the pupae of a coleopteran insect, *Tenebrio molitor* (Sláma, 2000). The similarity is curious as the pupae of *Cydia* and *Tenebrio* have profoundly different anatomical and morphological structures. The former pupae show a true lepidopteran type with all appendages fixed and covered by the common external case (*pupa oblecta*). By contrast, the pupae of *Tenebrio* represent the most common type, which is widespread among many insect orders (Coleoptera, Hymenoptera, Diptera, Neuroptera, for instance). It is characterized by freely extending appendages outside the body and by far more flexible abdominal segments (*pupa libera*). In spite of these morphological and taxonomical differences, the similarity in regulation of circulatory and respiratory functions between a lepidopteran *Cydia* and a coleopteran *Tenebrio* is striking. Thus, for example: (a) *Cydia* and *Tenebrio* pupae both exhibit the purely continuous type of respiration without remarkable bursts in the production of CO₂; (b) both exhibit regular heartbeat reversals with shorter, forward oriented (anterograde) and longer, backward oriented (retrograde) peristaltic waves; (c) in both cases there are periodically repeated EHP during the whole duration of the pupal instar; (d) the internal mechanisms regulating the cardiac (heartbeat) and extracardiac (EHP) pulsations are in both cases absolutely independent from each other for a major part of the pupal instar; (e) the frequency of EHP and also their time distribution are similar to the anterograde heartbeat; (f) during advanced pharate adult development, the cardiac and extracardiac pulsations are continuous and occasionally they pulsate in concert, which considerably enhances the flow of hemolymph; (g) in both species the fastest hemolymph flow has been associated with the anterograde heartbeat.

The functions of pupal heart have been recently studied in giant pupae of the tobacco hornworm, *Manduca sexta* by Smits et al. (2000). They used an invasive, conductometric method with implanted wire electrodes. As far as the pupal stage is concerned, they found only an ‘unpredictable’ heartbeat reversal without further specifications. More recently, the heartbeat patterns in diapausing pupae of *Manduca* have been studied by similar, noninvasive, thermographic techniques which have been also used in this study. It appears that these giant pupae exhibit a constant, regular and quite predictable switchovers between the anterograde and the retrograde cardiac pulsations (Sláma and Miller, 2001). However, the circulatory features of these diapausing pupae are substantially different from the above-mentioned (a)–(g) points that are valid for the pupae of *Cydia* and *Tenebrio*. Major differences in this respect are complete absence of EHP and large discontinuous bursts of CO₂ in *Manduca*. Nevertheless, these differences in circulatory functions between the giant diapausing pupae of *Manduca* and small developing pupae of *Cydia* do not seem to be causally related to different size of the body, because the developing pupae of *Manduca* show more or less similar circulatory and respiratory patterns as described here for *Cydia* and *Tenebrio*, e.g. more or less continuous respiration with frequent EHP and frequent heartbeat reversals (Sláma and Miller, 2001).

According to our calculations, the actual ventilatory effectiveness of the EHP alone would not be sufficient in *Cydia* to cover all pupal demands for oxygen. Based on common shape and dimensions of an average pupa, we can easily calculate a corresponding change of internal body volume that would be associated with 1 μm of abdominal contraction. The calculated ratio is that 1 μm of abdominal movement would cause 1.8 nl change of internal volume. Based on this ratio, we can calculate that the overall ventilatory effect of EHP is some 30 μl of air exchanged through the second abdominal spiracles in one hour. However, the data in Fig. 13 show that the investigated pupa consumed in one hour 28 μl of pure O₂, for which amount it required to transport at least 144 μl of air. We can further calculate that, in addition to 28 μl contributed by the EHP, the three sudden abdominal contractions per minute could further increase the ventilatory capacity of the body for 36 μl of air per hour, although this would still not be enough to cover all pupal demands for at least 144 μl of air per hour. Most of the remaining volumes could be supplied by occasional, large abdominal rotations, which have been always associated with the most extensive ventilatory effects or, eventually, a small amount could also be attained by trans-spiracular diffusion.

The results of simultaneous recordings of the heartbeat and abdominal movements (Figs. 4–7) showed, similarly to the case in *Tenebrio* (Sláma, 2000), that the actual impact of the heartbeat on abdominal movement

in *Cydia* is very small, some 20- to 50-fold smaller in comparison with that exerted by the EHP. We are thus inclined to believe that heartbeat itself is too weak to play any significant role in tracheal ventilation in small insects such as *Cydia*. This conclusion can be further supported by results obtained in pupae of some other species (Sláma 1999, 2000). It does not favor the idea that the insect dorsal vessel could exert pronounced ventilatory functions (Wasserthal, 1976, 1981, 1996).

The above-described calculations based on the convective bulk flow of air through insect spiracles would not be reliable without considering also the factors associated with physical diffusion of respiratory gases. These factors actually used to be the main subject of previous respirometric calculations (Krogh, 1920; Buck, 1962; however, see the diffusion-convection calculations of Kestler, 1985). It cannot be denied that the reciprocal diffusion of O₂ and CO₂ through the spiracles could be important specifically during the relatively short period of advanced pharate stage before adult eclosion. The techniques never revealed a complete constriction of the spiracles during this period of substantially increased metabolic intensity (absence of passive suction pattern). On the other hand, the techniques revealed at this time almost uninterrupted hemocoelic pulsations of different kinds (heartbeat, EHP and pulsation of the ventral diaphragm; see Sláma, 1999), suggesting the presence of actively regulated ventilation.

As already mentioned, the general rules of tracheal gas diffusion, which were elaborated by Krogh as early as in 1920, are certainly valid for the transport of gas within the closed pupal case. We have found, however, that as far as the respiratory gas exchange between the body and the environment is concerned, evolution replaced the principles of passive gas diffusion by the regulated ventilatory airflow. Krogh (1920) based his theory on a faulty assumption that immobile insect stages did not show any ventilatory movements. Of course, he could not see them by the naked eye at that time. Electronic methods that we have used document existence of at least three different kinds of the actively regulated abdominal ventilatory movements, most of which are not perceptible by the naked eye. Moreover, the results obtained with the young pupae of *Cydia*, which tightly close all spiracles and thus become completely isolated from the environment for at least 88% of total time, illustrates very well the absurdity of previous diffusional calculations. At this point one could ask a question about what could actually be the selective evolutionary advantage of active mechanical ventilation for insect life and development? The closed spiracles obviously minimize or abolish the respiratory water loss, which is imperative for all terrestrial organisms. The present demonstration of passive suction respiration in young pupae of *Cydia* shows for the first time, that a small insect (that is perhaps best suited for a diffusional

gas exchange) is able to close all spiracles for long periods of time, just like the giant lepidopteran pupae.

The results of anemometric recordings (Figs. 11 and 12) provide new information on respiration of insects of small size. They confirm previous observations of Sláma (1988) that insects can open or close spiracular valves in millisecond intervals of time and so they could regulate unidirectional inspirations or expirations of air through individual spiracles. This reveals a physiological concert of events that cannot be understood without anticipation of the corresponding nervous pathways (autonomic coelopulse system) that decide which spiracles will function at what moment (Sláma 1988, 1999). The results in Fig. 12 show that the endogenous nervous system of the pupa, in order to achieve the unidirectional filtration of air stream during extracardiac pulsation of 1 Hz, needs to operate spiracular valves of the second abdominal segment with the open–close velocity corresponding to 2 Hz. According to our measurements, the frequencies of both the cardiac and extracardiac pulsations progressively slow down towards the end of each pulsation. Yet the data in Figs. 11 and 12 show unidirectional filtration and perfect synchronization of spiracular openings with contractions of abdominal muscles at the beginning as well as at the end of EHP. This indicates that the responsible nervous system of the pupa (coelopulse) is able to coordinate perfectly the moments of spiracular valve openings with the movements of the abdominal pressure pump. Existence of such coordinated ‘accordion-type respiration’ has been suggested earlier by Sláma (1988). By contrast, existence of the mysterious phenomenon of ‘spiracular fluttering’ (Miller, 1974; Kestler, 1985; Lighton, 1996) has never been experimentally confirmed.

The discontinuous CO₂ release has been described in the literature many times (Brockway and Schneiderman, 1967; see reviews by Buck, 1962; Miller, 1974; Kestler, 1985; Lighton, 1996) although relatively little attention has been paid so far to continuous release of CO₂ from the body. The current theories still assume a purely diffusive inflow of O₂ and reciprocal diffusion of CO₂ through the spiracles (Buck, 1962; Kestler, 1985). As we have seen, this does not apply to young pupae of *Cydia*, which keep the spiracular valves closed. When they pulse open, a bulk flow of air flushes into the body against the negative gradient of pressure. In this case, the gas exchange needs to be actively regulated and the motor of this regulation probably depends on the periodically repeated, inconspicuous EHP, as in the case of *Tenebrio* (Sláma, 2000).

Adequate circulation of hemolymph represents an acute problem not only in large, but also in small pupae of *Cydia*. Hemolymph circulation seems to be sufficiently well executed by periodic reversals of the heartbeat within the central hemocoelic body cavity (Figs. 2–7). Major obstacles are concerned with circulation of the hemolymph through long pupal appendages. These are

mostly tubular structures that are located far from reach of the dorsal vessel. Adult stages of insects contain various accessory pulsatile organs, which help to pump the hemolymph through antennae, palpi, legs and wings. However, the last review on accessory pulsatile organs of insects (Pass, 2000) does not contain a single report concerning possible existence of these organs in the pupal stage of insects. Accordingly, we have been also unable to find independent pulsatile organs in the appendages of *Cydia* pupae. However, one can observe conspicuous movements of internal membranes through the transparent cuticle of the wing lobes. These movements have been correlated with EHP, not with the heartbeat. We may thus assume that, like in the case of *Tenebrio* pupae (Sláma, 1999), circulation of hemolymph through the pupal appendages is realized chiefly through EHP and sudden changes in hemocoelic pressure. This possibility is now being further investigated.

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