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Calling Behavior Modulates Heartbeat Reversal Rhythm in the Silkmoth *Bombyx mori*

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ABSTRACT—A calling female of *Bombyx mori* every several seconds lifts the abdomen to extrude pheromone glands. The amplitude of the abdominal movement changes with alternation in the flow of haemolymph due to rhythmic heartbeat reversal. The first forward beating pulse usually occurs at the lowered position of the abdomen and subsequent forward beating pulses are often inhibited by elevation of the abdomen. During a calling period in the photophase or in the early scotophase, the duration of backward beat phases is significantly longer than that of forward beat ones. When a female terminates calling behavior in the late photophase and the late scotophase, backward phases become shorter and forward phases become longer. Similar changes in length of the forward and backward beat phases occur after elimination of calling behavior by mating and by transection of the ventral nerve cord. These observations suggest peripheral modulation of heartbeat reversal rhythm. The longer duration of the backward beat phases may be suitable to maintain the calling posture and for efficient transport of metabolites and tracheal ventilation in the abdomen with a high rate of metabolism during calling behavior.

INTRODUCTION

The heart of insects is a tubular vessel running along the dorsal midline. Cyclic alternations in the direction of peristaltic movement of the heart have been observed in many orders of insects (Gerould, 1933; Jones, 1964; McCann, 1970; Wasserthal, 1996) and the heartbeat reversal causes periodic movement of haemolymph between thorax and abdomen (Wasserthal, 1980, 1981). Cardiac activity of insects is under the control of a number of factors: studies on these factors usually focused on the heartbeat rate (Jones, 1974). For an insect with a heartbeat reversal mechanism, the duration and direction of heartbeat or the rhythmicity of reversal may also be important for tracheal ventilation, regulation of hydrostatic pressure in the haemocoel and transport of metabolites, hormones and heat (Wasserthal, 1980, 1981, 1996). Although a heartbeat reversal rhythm appears to be determined by intrinsic pacemaker activities of the heart (Gerould, 1929; McCann, 1970; Angioy and Pietra, 1995), neural control of reversal is implicated by appearance of a prolonged phase of heartbeat in a particular direction during wing spreading of a newly emerged moth (Queinnec and Campan, 1972; Moreau, 1974; Tublitz and Truman, 1985; Wasserthal, 1996) and after a flight activity in the hawk moth (Heinrich, 1971). Inhibitory action of locomotor activity on particular phase of heartbeat in the blowfly also suggests neural modulation of endogenous heartbeat reversal (Thon, 1982).

Female moths extrude the pheromone gland and emit sex pheromone into an air stream to attract males. A silkmoth *Bombyx mori* in a calling posture contracts the dorsal and lateral musculatures of the body wall and bends the abdomen upward at an interval of several seconds. The abdominal movement often shows a slow change in amplitude and the change is highly coordinated with heartbeat reversal rhythms (Ichikawa, 1998). Accumulation of haemolymph in the abdomen during a backward phase of heartbeat seems to enhance abdominal movement and the activity of a neurosecretory system controlling sex pheromone production in *Bombyx mori* (Ichikawa, 1998).

We report here that periodicity of heartbeat reversal in a calling female *B. mori* is strongly modulated by abdominal movement and this modulation may be significant for blood circulation to support calling motor activity

MATERIALS AND METHODS

Commercially available F1 hybrid of *B. mori* (Kinshu × Showa or Shunrei × Shogetsu) were used. Pupae and adult moths were placed at 26±1°C under a 16 hr light/8 hr dark photoperiod. Some females were mated with an intact (fertile) male.

Abdominal movements and electrocardiograms (ECGs) were recorded from females under a restrained or unrestrained conditions. After a newly emerged female was immobilized in crushed ice for 15 min, a piece of Teflon-coated silver wire (0.1 mm in outer diameter) for recording ECGs was placed near the dorsal loop of the aorta and another one was inserted into the anterior portion of the thorax. Wounds were sealed with a quick-drying glue. The female was perched on a platform placed at an angle of 45°. To monitor abdominal movement, the 4th or 5th abdominal segment of the female was placed

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between a pair of infrared light emitting diodes and a phototransistor. This unrestrained animal immediately started and continued calling behavior without moving from the platform during the calling period but usually escaped from the place after termination of calling behavior. Therefore, most recordings of abdominal movements were made on restrained females. After removing all legs of a female, the ventral part of the thorax and wings were fixed to the platform with paraffin. The female usually struggled to get free from restraint for a few hours and started to call normally. There were no significant differences in patterns of calling behavior and cardiac activity between restrained and unrestrained animals. Output of the phototransistor was practically linear within a dynamic range of 5 mm. All electrical signals were amplified, digitized and stored on a computer equipped with an A/D converter 〔MacLab 8 s (AD instruments) or 1401 plus (Cambridge Electronic Design)〕. Signals were usually filtered 30 Hz and digitized at 100 or 200 Hz. During chronic recordings, the animal was illuminated at the same LD cycles, using a fluorescent lamp. Light intensity was about 100 lux.

Before transection of the ventral nerve cord (VNC), a female was immobilized (anesthetized) in crushed ice for 15 min. The VNC between the thoracic ganglion and the abdominal ganglion was transected using microscissors after removing part of the cuticle above the cord. After sealing the wound with paraffin, the female was kept at the room temperature for about 15 min to recover from anesthesia.

RESULTS

Abdominal movements and ECGs were monitored on a female for 3–5 continuous days under the 16 hour light/8 hour dark photoperiod. A virgin female usually went through two calling periods in a day: the first period began at onset of the photoperiod and continued for 10–13 hr; the second one began at the onset of scotophase and lasted for a few hours. Visual inspection of peristaltic waves of the heart through a transparent cuticle during an ECG recording confirmed that a phase of the cardiac activity with a higher pulse rate (tachycardia) corresponded to a forward beating period and that with a lower pulse rate corresponded to a backward beating period (see Fig. 1). Because there was little consistent change in pulse rates over a long period of time (hours or a day) among different females, lengths of individual heartbeat periods were analyzed.

Calling patterns and heartbeat reversal

There were large variations in the temporal pattern of calling behavior among 16 1-day-old females. Five females showed oscillatory movements of similar amplitudes throughout a calling period, although a slight decrease in the amplitude and/or an increase in the irregularity of motions were often distinguished during a forward beating period (Fig. 1A). Nine females usually showed a large (*ca.* 30–80%) attenuation in the amplitude of the motion during a forward period (Fig. 1B). Such attenuation of abdominal movements often increased with age of the moths. In the remaining two females, abdominal movements were apparently intermittent: the amplitude of motion rapidly decreased to near zero during a forward beating period and such a motionless phase continued until abdominal movements resumed at an early or a middle phase of the backward beating period (Fig. 1C). When waveforms of abdominal movement of the second type during different cycles of backward/forward beating periods were averaged after setting time of the first forward pulses to time 0, the averaged position of the abdomen apparently changed with heartbeat reversal rhythms (Fig. 2). The averaged abdominal position gradually elevated during a backward period to become maximal just before the reversal and lowered thereafter until forward beats terminate. There is a steep valley at the timing of heartbeat reversal, because the first forward pulse usually occurs at the lowered position of the abdomen (see Fig. 3).

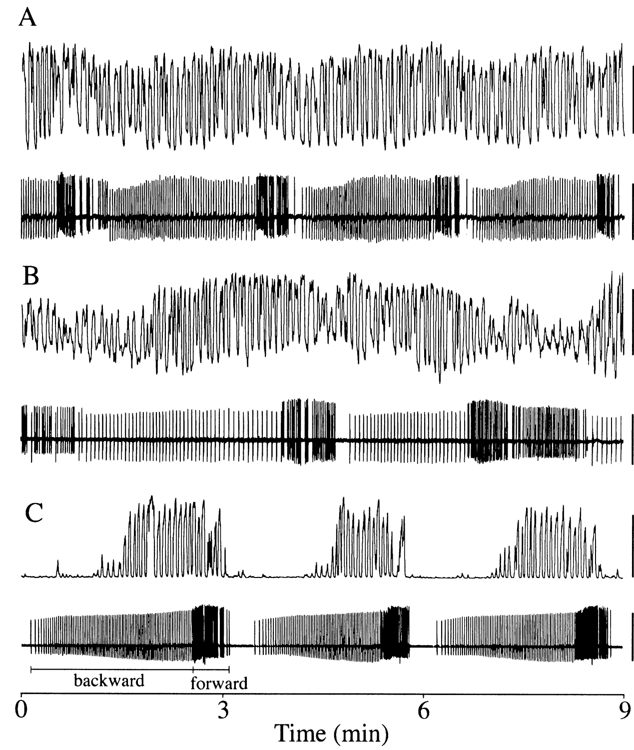

Fig. 1. Typical patterns of calling behavior and its relation to the heartbeat reversal rhythm. A, B and C were recorded from 1 day-old different females at the middle photophase. Each record shows abdominal motions (top) and an electrocardiogram indicating periodic heartbeat reversals (bottom). A forward beat period distinguishable from a preceding backward beat period by a higher pulse frequency (tachycardia). (A) Regular abdominal movement throughout forward and backward beat periods. (B) Fluctuated abdominal movement with a large attenuation in the amplitude of motions during a forward beat period. (C) Intermittent abdominal movement with a motionless phase. Scale bars: 2 mm (top) and 0.2 mV (bottom).

Effect of calling behavior on forward beating

Fig. 4 shows the timing of the first forward beating pulses determined as relative phase of abdominal motions. The first pulses occur around a middle point between two upward motions. A brief pause or termination of forward beat was observed in response to a large upward motion (Fig. 3, see also Fig. 1). Furthermore, there was a large difference in the length of forward and backward periods, under different conditions of behavior. Backward beats were dominant during a calling period (Fig. 5A), but were no longer dominant during a resting

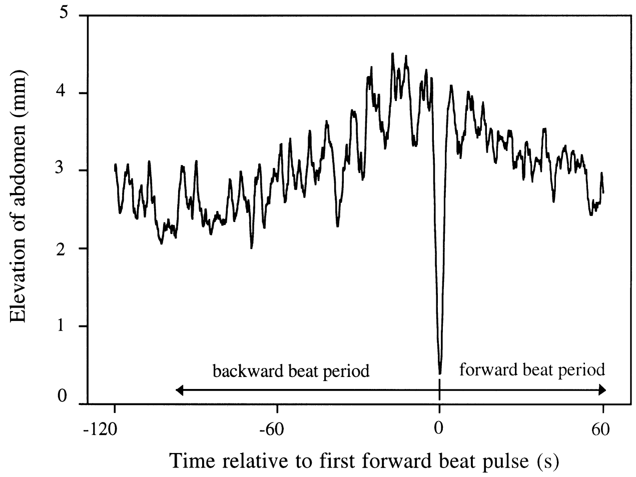

Fig. 2. Changes in average position of the abdomen due to alternation in blood flow. Waveforms of the abdominal movement during 100 cycles of forward/backward beat were averaged after setting time of the first forward pulse of each cycle to time 0.

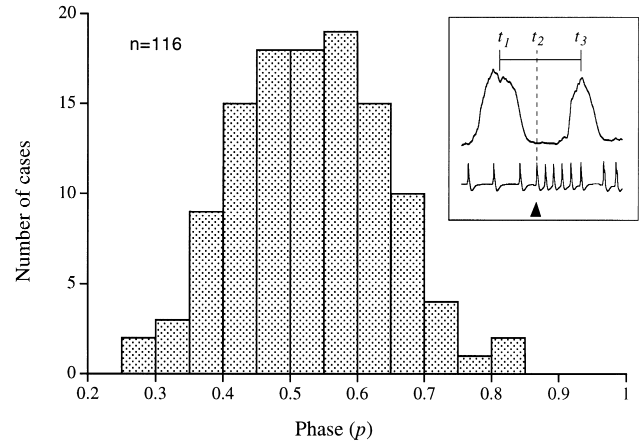

Fig. 4. Phase relationship between abdominal motion and heartbeat reversal. Inset illustrates the phase (p) of the first forward beat pulse (arrowhead, t_2) which is defined as $p = (t_2 - t_1)/(t_3 - t_1)$, where t_1 and t_3 are the time of the preceding and next upward motions, respectively.

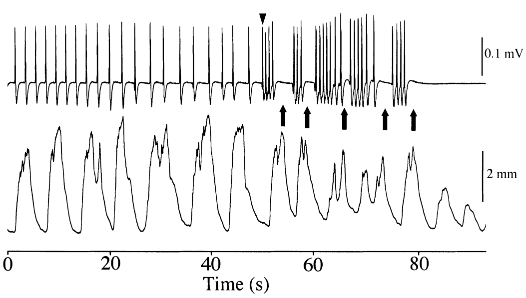

Fig. 3. Inhibition of forward beat pulses by abdominal motions. The first forward beat pulse (arrowhead) occurs at a lowered position of the abdomen and forward beats are often interrupted by elevation of the abdomen (arrows).

(non-calling) period (Fig. 5B). After termination of calling behavior, backward beating periods usually became shorter whereas forward periods became longer; a sequence of forward beats sometimes started without any preceding backward beat after a brief pause (Fig. 5B). A close relationship between abdominal movement and length of forward and backward periods was observed at the last phase of a calling period when abdominal movement often became intermittent (Fig. 6). When a series of large abdominal motion occurred, backward beats became dominant, though there were marked fluctuations in the length of heartbeat periods.

Effect of transection of ventral nerve cord and mating on reversal rhythm

The findings described above suggest that an elevation

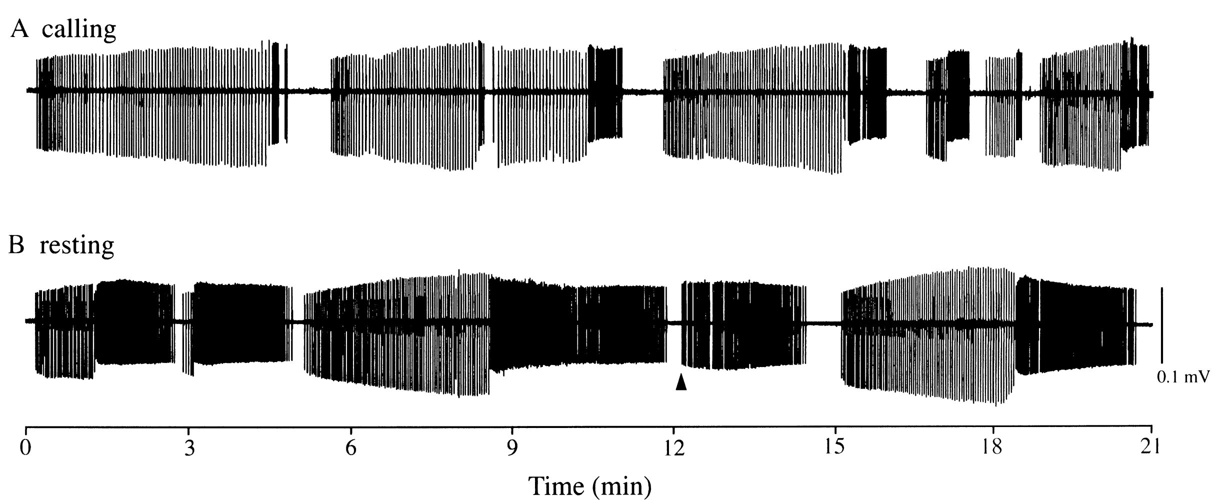

Fig. 5. Electrocardiograms recorded from a virgin female during a calling (A) and a resting period (B). A long forward beat phase sometimes begins with no preceding backward beat (arrowhead).

of the abdomen may inhibit the generation of forward beat. Because transection of the ventral nerve cord (VNC) made the abdomen immobile, effects of abdominal movement on heartbeat were examined by comparing activity patterns of the heart before and after the VNC transection. The VNC of a virgin female was cut at about midpoint of the calling period during the photophase (Fig. 7). The activity pattern of the heart before transection was characterized by prolonged backward periods, short forward periods and large variability in the length of both periods. A period of backward beating sometimes lasted more than 10 min. Mean±SD of the duration of backward beating for 2 hr before VNC transection was 309.1±180.2 sec and that of forward beating was 6.2±7.7 sec. After VNC transection, forward beat periods significantly lengthened and backward ones were shortened. The longest forward period before VNC transection appeared most common after VNC transection. The forward beating pattern became regular without a brief pulse stop as a sign of inhibition by abdominal movement and the pulse rate gradually decreased until heartbeat usually paused (see inset of Fig. 7). Mean±SD sec for 2 hr after VNC transection were 135.4±58.9 sec (Backward period) and 31.5±6.8 sec (forward period). Thus, the VNC transection shortened the backward period by a factor of 0.44 and lengthened forward period by a factor of 5.1. A decrease in variability in the duration of forward and backward beat phases is apparent by comparing the ratios SD/mean before and after VNC transection. Among six females, values of the shortening rates were 0.29–0.62 (mean±SD = 0.46±0.12) and prolongation rates were 2.3–5.7 (mean±SD = 4.0±1.1).

The VNC transection could be replaced by mating. Fe-

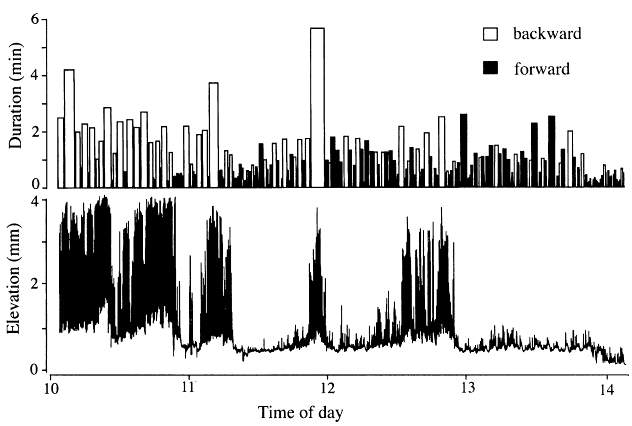

Fig. 6. Relationship between abdominal movement and duration of forward and backward beat periods. Backward beat becomes dominant when transient calling behavior occurs at the last phase of a calling period.

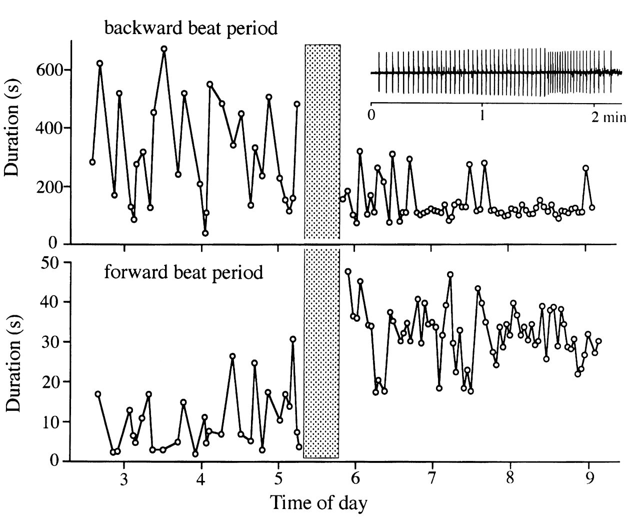

Fig. 7. Effect of transection of the ventral nerve cord on heartbeat reversal rhythm. Shaded area indicates the period for anesthesia, transection of the nerve cord and recovery from the anesthesia. Inset shows a cycle of backward/forward beat after VNC transection.

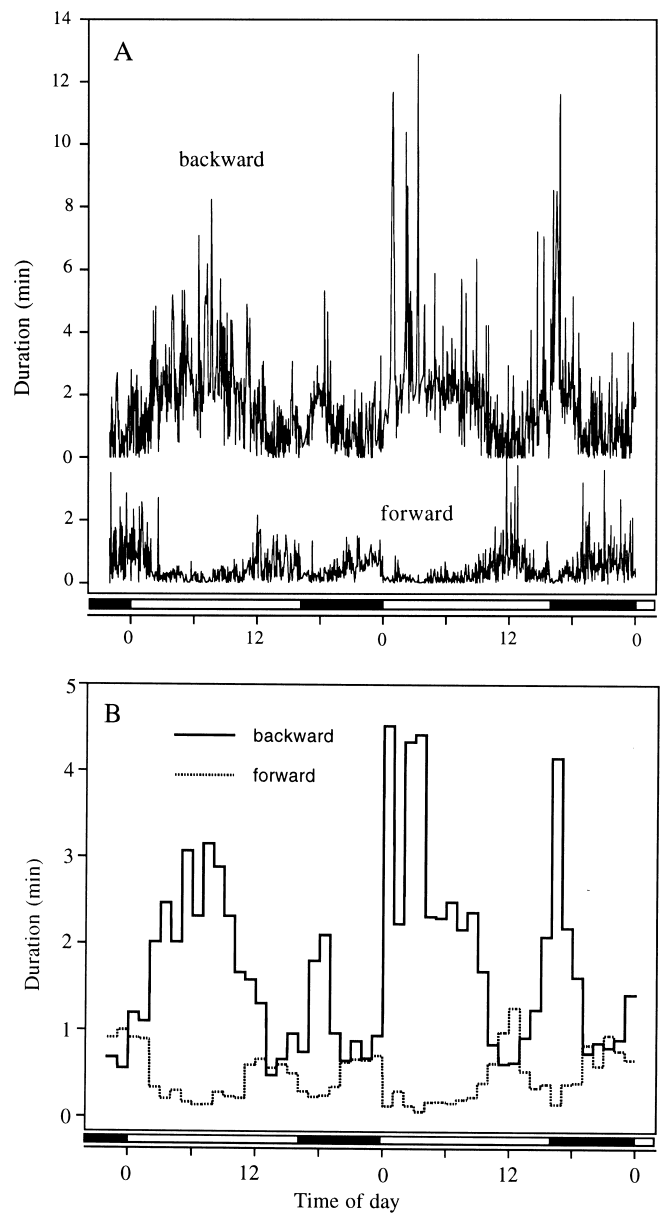

Fig. 8. Daily changes in the duration of individual forward and backward beat periods (A) and averaged duration (B) in a virgin female.

males terminated calling behavior as soon as a fertile male made contact. The cardiac activity pattern after the start of mating was indistinguishable from that of a virgin female with a transected VNC. Among six mated females, values of the shortening rates for backward periods were 0.23–0.95 (mean±SD = 0.58±0.29) and prolongation rates for forward periods were 2.3–8.0 (mean±SD = 4.1±1.8).

Daily change in reversal rhythm in a virgin and mated female

Long term chronic recordings of ECGs were made under unrestricted conditions. The time series of durations of single forward and backward beat periods showed a large fluctuation superimposing on a daily component (Fig. 8A). When the durations were averaged every one hour, the daily component was apparent and coincided with the daily change in calling behavior (Fig. 8B). Average durations of forward periods generally shortened as those of backward phases become longer, or vice versa. Such a daily change in cardiac activity and predominance of backward beats disappeared after mating with a fertile male (Fig. 9).

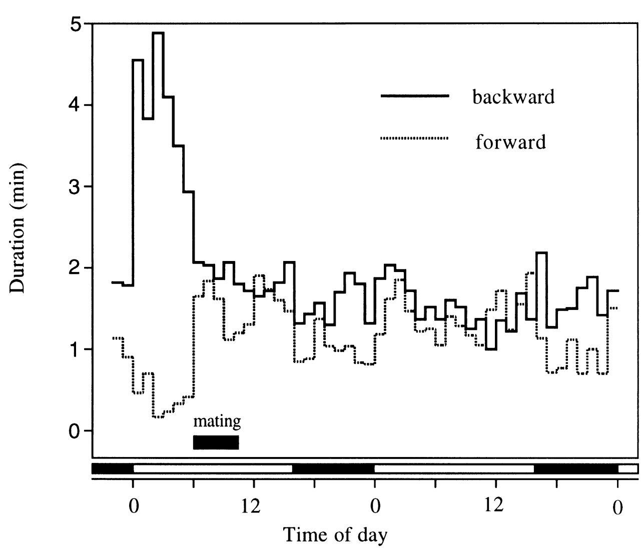

Fig. 9. Loss of apparent daily change in averaged duration of forward and backward beat periods after mating.

DISCUSSION

Two types of ventilatory abdominal movements were observed in the butterfly *Papilio machaon* and the giant silkmoth *Attacus atlas* (Wasserthal, 1980,1981): slow contractions of the abdomen coordinated with periodic heartbeat reversal and fast peristaltic movements over the abdomen. The slow contraction induce periodic changes in abdominal length, the pattern of which resembles the change in average (basal) position of the abdomen in *B. mori* (Fig. 2): an expansion of the abdomen in the former corresponds to an elevation of the abdomen in the latter. The fast ventilatory movements in *Attacus* occur during backward beating periods and have a frequency of 6-10 min^{-1} at 22°C, the frequency is practically the same as the frequency of calling abdominal motions, 8–15 min^{-1} observed in *Bombyx* at 26°C (Ichikawa, 1998). Thus, abdominal movement in *Bombyx* during calling may be a kind of the ventilatory abdominal movement or its variation.

The coordination of cardiac activity with abdominal movement (Figs. 1 and 2) suggests that the heartbeat reversal rhythm may affect the calling behavior by inducing periodic accumulation/removal of haemolymph in the abdomen. The abdomen increases its volume by the inflow of haemolymph and bends upward maximally to perform a large complete motion when the dorsal and lateral musculatures of the body wall contract. Conversely, the calling behavior apparently affects the cardiac activity, because ECGs from a calling female indicate an inhibition of forward beating in response to upward abdominal motion (Fig. 3). A variety of sensory stimulation induces many types of cardiac responses and it is often effective during a forward beat phase. Heartbeat reversal from forward to backward direction is easily induced by visual, olfactory and taste stimulation in the blowfly (Campan, 1972; Angioy *et al.*, 1987; Angioy, 1988). Spontaneous movement of the genitalia in the cranefly inhibits forward beats and reduces the length of the forward period from an average 60 beats to about 30 beats (Gerould, 1933). Free locomotor activity in the blowfly inhibits forward beats and shortens the sequence of forward beats: the mean duration of sequences (*ca.* 6 sec) in a free walking fly is significantly shorter than that (*ca.* 15 sec) in a restrained fly with waxed legs and wings (Thon, 1982). Results in the present study suggest two modes of the inhibitory action of abdominal movement on cardiac activity in a calling silkmoth: (1) an upward abdominal motion inhibits forward beats and facilitates earlier termination of a forward beating phase, (2) elevation of the abdomen limits or gates the timing of heartbeat reversal from backward to forward beating (Figs. 3 and 4). A relatively short duration of backward periods at a state without calling behavior (Figs. 5–9) suggests that the second mode of action may, at least in part, function in prolonging backward periods during calling. This prolongation may be due to a positive feedback loop: accumulation of haemolymph in the abdomen during a prolonged backward period may elevate a basic abdominal position, elevation of the abdomen then delays the initiation of forward beat, and the delay of forward beat initiation in turn facilitates haemolymph accumulation in the abdomen. Changes in duration of backward phases were not evident in the cranefly and blowfly. The identification of a putative receptor and a neural pathway mediating the inhibitory action of spontaneous motor activities on cardiac activity remains to be studied. A moth has abdominal stretch receptors associated with intersegmental receptor muscles which monitor stretch or posture of the abdomen (Finlayson and Lowenstein, 1958). Such a proprioceptor may mediate the inhibitory action of calling abdominal movement. Because the abdominal movement appears to modulate activity of a neurosecretory cell system in the brain-suboesophageal ganglion complex (Ichikawa. 1998), a signal from the putative receptor possibly runs up the VNC to the brain and enters the cardiac nervous system originating in the frontal ganglion (Ai and Kuwasawa. 1995).

A characteristic feature of the cardiac activity of *B. mori* is that durations of forward and backward periods fluctuate largely (Figs. 5–8). Similar fluctuations are seen in the ECGs from many insect hearts at various stages of development (Wasserthal, 1996). When SD/mean of the duration of forward and backward beating was calculated as a simple measure of fluctuation, the ratios usually decreased after elimination of abdominal movement by VNC transection (Fig. 7) or mating. A large fluctuation during calling may be due to an inhibitory effect of the abdominal motion: gating the timing of reversal from backward to forward within a particular position of the abdomen (Fig. 4) and facilitating termination of a forward beat phase (Fig. 3) may make each phase of heartbeat variable and increase irregularity of the reversal rhythm. Other factors such as spontaneous visceral movements possibly disturb cyclic cardiac activity (Jones, 1974).

Heartbeat reversal occurs in a number of insects at various stages of development. Each end of the dorsal vessel has a myogenic pacemaker activity and interactions between two pacemakers is thought to determine a basic reversal rhythm (Gerould, 1929; Tenney, 1953; McCann, 1970; Angioy and Pietra, 1995). In *B. mori*, spontaneous reversal is seen at all stages after wondering stage (Gerould, 1929). Ai and Kuwasawa (1995) noted neural pathways for cardiac reflexes triggered by mechanical stimulation in the larvae. There are three known major pathways mediating the cardiac responses: (1) anterior and posterior cardiac nerves arising from the frontal ganglion, (2) dorsal nerves of the 1st abdominal ganglion and (3) dorsal nerves of the 7th abdominal ganglion. Because stimulation of anterior and posterior cardiac nerves inhibits forward beat and trigger backward beat (Ai and Kuwasawa, 1995), the frontal ganglion may be a putative center for neural modulation of periodic reversal of heartbeat. However, decapitation (or removal of the frontal ganglion) does not prevent or seriously affect heartbeat reversal (Gerould, 1933; Tenney. 1953). In a preliminary experiment using adult *Bombyx*, we observed that after removal of the terminal abdominal ganglion (TAG), a normal heartbeat reversal was replaced by continuous backward beats or a rapid alternation of single backward and forward pulses. This finding suggests that there is a great deal of central nervous control of heartbeat reversal in the adult moth and that the TAG and the third pathway may play a crucial role in maintenance of a basic reversal rhythm that appears during a non-calling period. Because dorsal nerves from the TAG innervate alary muscles of the dorsal vessel at 7th and 8th abdominal segments, the site where a caudal pacemaker of heartbeat seems to localize to produce forward heartbeat (Tenney, 1953; Angioy and Pietra, 1995; McCann, 1970), removal of TAG or severance of the nerves may drastically alter pacemaker activity. The dorsal vessel with an intrinsic periodic activity and its nervous control mechanism may consist of a “complex system”. An example of complexity of the cardiac system appears to be represented by several different types of heartbeat reversals, including alternation of single forward and backward beats observed in the moth *Telea polyphemus* (Tenney, 1953). Characterization of such a system is the subject in the future.

Insects hearts, in cooperation with accessory pulsatile organs and ventral diaphragm, regulate circulation of haemolymph that is essential to maintain hydrostatic pressure in the haemocoel and for transport of hormones, nutrients, wastes and heat. For an insect with a heartbeat reversal mechanism, duration of a heartbeat phase is an important factor for distribution of haemolymph. Prolonged forward beat with an accelerated pulse rate continues during wing spreading of newly emerged moths (Queinnec and Campan, 1972; Moreau, 1974; Tublitz and Trumam, 1985), and it is essential for complete inflation of wings in a giant silkmoth (Wasserthal, 1996). Continuous forward beats after flight activity facilitates cooling down of the thorax temperature in *Manduca sexta* (Heinrich, 1971). Ichikawa (1998) suggested that prolong backward flow of haemolymph may be suitable for rapid delivery of concentrated pheromonotropic neuropeptides released in the head to the sex pheromone gland in the abdomen in the silkmoth. A calling female of *B. mori* lifts its heavy abdomen filled with many eggs by contracting the lateral and dorsal musculatures of the body walls. The long duration of the backward periods may be needed for a sufficient supply of haemolymph to the abdomen with a higher metabolic rate caused by a strong muscle activity, as well as for an increase in abdominal hydrostatic pressure needed for extrusion of the pheromone gland. Because tracheal ventilation (CO_2 emission) is closely related to periodic heartbeat reversal and abdominal movement (Wasserthal, 1980, 1981, 1996), large abdominal motions during a long period of backward beating may facilitate ventilation of the abdomen.

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