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DEVELOPMENTAL CHANGES IN *IN VIVO* CARDIAC PERFORMANCE IN THE MOTH *MANDUCA SEXTA*

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Summary

While an extensive literature on cardiovascular development exists for insects, almost all studies focus on *in vitro* preparations, and very few report on more than a single developmental stage. The present study examines *in vivo* cardiac performance in the intact, unanesthetized larvae, pupae and adults of the tobacco hornworm *Manduca sexta*. For all three stages, electrode pairs of fine steel wire were inserted subcuticularly at two dorsal abdominal locations. Impedance signals produced by contraction of the dorsal abdominal vessel (tube heart) were amplified and recorded. In addition to providing heart rate, a comparison of the relative timing of the signal from each electrode pair allowed the calculation of the propagation velocity and direction of heart contraction. Experimental treatments of intact animals included exposure to hypoxia and hyperoxia (21 %, 15 %, 10 %, 5 %, 0 % and 100 % O_2), to hypercapnia (0 %, 4 %, 8 %, 16 %, 20 % and 24 % CO_2), to temperature variation (10, 20 and 30 °C) and to 2 min periods of forced activity.

The pattern of contraction of the dorsal abdominal vessel of *M. sexta* changed substantially with developmental stage. Larvae showed a relatively simple, invariably posterior-to-anterior pattern (mean rate 34.8±1.16 beats min^{-1}). The heart rate pattern in pupal *M. sexta* displayed great variability in rate, amplitude and direction. Periods of regular heart beats (21.5±1.09 beats min^{-1}) were frequently and irregularly interrupted by periods of cardiac arrests ranging from a few seconds to over 20 min. Adults showed a highly stereotypic but complex pattern, with periods of 'fast forward' (FF; rate 47.6±2.6 beats min^{-1}), 'slow forward' (SL; 32.8±3.0 beats min^{-1}) and 'reversed' (R; 32.2±2.4 beats min^{-1}) beating. The contraction propagation velocity in larvae and pupae averaged 5.52±0.36 and 2.03±0.11 cm s^{-1}, respectively. The SF, R and FF phases of the adults had average propagation velocities of 5.52±0.51, 5.05±0.52 and 5.43±0.37 cm s^{-1}, respectively.

Heart rate and contraction propagation velocity were remarkably resistant to ambient hypoxia and hypercapnia at all developmental stages, decreasing significantly only at 0 % O_2 or 24 % CO_2. As expected, the heart rates of all three developmental stages increased significantly with increasing temperature, with heart rate Q_{10} values for larvae, pupae and adults of 2.33, 3.14 and 1.61, respectively, between 10 and 20 °C. Corresponding Q_{10} values for these stages between 20 and 30 °C were 2.22, 2.03 and 2.29.

Larval heart rates showed no significant response to forced activity induced by prodding. In contrast, adult heart rate increased nearly fivefold from 50.1 beats min^{-1} during rest to 223.5 beats min^{-1} after 1 min of prodding. The activity-induced tachycardia in adults ceased within 10–12 min.

Patterns of cardiac contraction in larval, pupal and adult *M. sexta* were as dissimilar as their morphological appearances and revealed a gradation from simple to complex. These developmentally based distinctive cardiac patterns are undoubtedly related to developmental differences in both morphology and life-style. Larvae are anatomically 'homogeneous' compared with other stages, with no distinct head, thorax and abdominal region (or wings) that might require selective perfusion or drainage. The far more complex pattern of heart activity seen in pupae probably relates to the dramatic changes in internal morphology during this stage. Simultaneous degradation and synthesis of tissues throughout the body may expose the heart to numerous peptides or neurohormones that affect cardiac activity. In adult moths, the complex and repetitive pattern of cardiac activity is reflected in the previously described complexity of hemolymph movement, together with thermoregulatory capabilities in this species that depend on well-regulated hemolymph movements between the thorax, wings and abdomen. Future studies on developmental changes in the control of heart rate in *M. sexta* and other insects should prove of great interest.

Key words: development, insect, *Manduca sexta*, larva, moth, pupa, heart rate, hypoxia, hypercapnia.

Introduction

Developmental physiology has burgeoned in the last decade, with many studies focusing on cardiovascular development as physiologists strive to understand how cardiovascular function begins, first becomes regulated, and then changes during subsequent development. Most attention has been focused on vertebrates, especially on a few key 'model' species such as the zebrafish *Danio rerio*, the African clawed frog *Xenopus laevis*, the chicken embryo, and fetal mammals such as the sheep (for references, see Burggren and Keller, 1997). Far less is known about invertebrates, especially those with a complex, relatively high-pressure circulation such as arthropods and molluscs (McMahon et al., 1997a,b; Reiber, 1997; Spicer, 1994). For invertebrates, perhaps the most extensive literature on the development of cardiovascular performance exists for the insects (see Miller, 1985; Tublitz, 1989; Tublitz et al., 1992; Ali and Kuwasawa, 1995). However, these studies characteristically either used *in vitro* preparations or focused only a single developmental stage rather than a developmental series. Consequently, surprisingly little is known about *in vivo* cardiovascular development in insects.

The ontogeny of heart form and performance in insects is fascinating because of the profound quantum changes that often characterize their development. In lepidopterans, for example, a larval caterpillar undergoes eclosion to form an external inactive pupa, from which eventually emerges an adult flying moth or butterfly. Each of these three stages differs greatly in behavior, in morphology and, we hypothesize, in physiology. In contrast, most vertebrates develop in a much more linear, progressive fashion with only small incremental changes over the course of each stage of development, i.e. they show a developmental continuum rather than a series of quantum changes. Even among other arthropods, in which developmental change occurs with defined instars, overall changes from instar to instar are typically relatively minor. By studying *in vivo* cardiovascular physiology in an animal that develops with large quantum steps, we are likely to determine whether external morphological changes are correlated with similar profound shifts in cardiac performance.

The present study examines *in vivo* cardiac performance in the intact, unanesthetized larvae, pupae and adults of the tobacco hornworm *Manduca sexta*. This leptodopteran is relatively large in all three major stages, which has allowed measurements to be made of the rate of contraction of the 'heart' (contractile dorsal vessel) and of the rate of propagation of the contractile wave along the heart in all three stages. The heart of many insects will periodically reverse direction, a phenomenon first described by Malpighi (1669) and subsequently studied by Gerould (1933), Wasserthal (1976) and others (see Miller, 1985). We have quantified the differences in heart rate during forward and reversed beating in *M. sexta* during development. Because the act of eclosion and associated burial under a substratum could lead to hypoxic or hypercapnic stress, we also examined the effects of these conditions on heart rate in all three developmental stages. Finally, by studying intact, non-restrained animals, we were able to investigate how activity alters heart performance. Collectively, these data present a picture of radical, development-based changes in the regulation and patterning of heart performance throughout the body of *M. sexta* as it develops from caterpillar to moth.

Materials and methods

Animals

Larvae, pupae and adult stages of the tobacco hornworm moth *Manduca sexta* L. were obtained from a long-established colony at the University of Massachusetts, Amherst. Body masses (±0.1 g) and ages (to the nearest day) were recorded for individuals within each stage at the time of experimentation. Larvae (instar V) varied in mass from 2.1 to 9.4 g, largely because of their age (10–21 days post-hatch). Pupae (2.5–5.7 g) were classified according to the number of days following pupation (1–14 days). Adult moths (0.8–1 g, 1–5 days post-emergence) were selected from reared pupae. Animals of all stages were maintained on a 12 h:12 h (L:D) light cycle and were tested between the daylight hours of 10:00 and 18:00 h.

Measurement techniques

Individuals of each developmental stage were anesthetized by brief exposure to 100 % CO_2 until reflex responses to touch ceased. Electrode pairs formed from fine steel wire (40 gauge) were carefully inserted approximately 1 mm beneath the cuticle at two dorsal locations on the abdomen (see below and Fig. 1). Insertion of each electrode was routinely associated with a tiny efflux of hemolymph, indicating that the electrode was in good contact with body fluids. After hemolymph coagulation had occurred, the electrode and insertion point were sealed with cyanoacrylate glue administered from the tip of an insect pin. In some larvae, in which coelomic pressure was elevated and highly influenced by body movements, a latex patch (0.5 mm^2) was glued over the site where the electrodes pierced the body.

Each pair of electrodes was positioned to span the dorsal vessel, which runs close to the dorsal body wall in the midline of all developmental stages. For pupae and adult moths, an anterior pair of electrodes was routinely placed on the first or second abdominal segment, while the posterior electrode pair was positioned at the penultimate abdominal segment (see Fig. 1). In larval moths (in which no distinct abdominal segment exists), anterior and posterior electrode pairs were placed approximately 20 mm apart, with the posterior pair anchored in the penultimate segment. Distances between the anterior and posterior electrode pairs were measured to the nearest millimeter.

Animals of all stages were allowed 12 h to recover from anesthesia and surgery. Subsequent maintenance of, and experiments with, the animals were performed while they remained unrestrained within 125 ml flasks containing moist paper towels. All animals were maintained at 20 °C, and all recordings were made at this temperature unless otherwise indicated. Humidified air or an experimental gas mixture (see

below) was continuously passed through the flask. Electrodes pairs were led from the top of the flask and were attached to impedance converters (UFI, Morro Bay, CA, USA; model 2991) at the start of an experiment. Impedance changes due to the contraction of the dorsal abdominal vessel were clearly detected at each electrode site; the size and frequency of these events were qualitatively recorded by a.c. output to a physiograph (Narco Biosystems, Houston, TX, USA). The timing of the contraction of the dorsal blood vessel at each electrode site was recorded on separate physiograph channels (Fig. 1). The output signal of one of the electrode pairs was connected to a biotachometer (Narco Biosystems) to generate instantaneous heart rate on a third physiograph channel. The propagation of contraction along the dorsal vessel (expressed as a velocity in $cm\,s^{-1}$) was determined for each individual by dividing the distance between the electrode pairs by the time between peak impedance waveforms at each electrode pair site (arrow in Fig. 1). The direction (anterior to posterior or *vice versa*) of the vessel contraction was determined, by comparing visual observations with high-speed tracings ($25\,mm\,s^{-1}$), on the basis of which electrode location contracted first. The amplitude of the impedance signal was not a reliable indicator of contractile strength (from visual observations), so no attempt was made to quantify amplitude changes of the dorsal vessel performance.

Hypoxia and hypercapnia

Following 1 h of of control measurements under resting, undisturbed conditions, each animal's cardiac responses to changes in ambient gas levels, body temperature and exercise were evaluated. Exposure of the whole animal to various levels of O_2 and CO_2 was accomplished by passing pre-mixed levels of these gases (Wöstoff gas-mixing pump, Bochum, Germany) into the holding flask at $250\,ml\,min^{-1}$. Each level of O_2 (21 %, 15 %, 10 %, 5 %, 0 %, 100 %, all gases with balance N_2) and CO_2 (0 %, 4 %, 8 %, 16 %, 20 %, 24 %, all gases with balance air) was presented to the animals serially in the stated order for 10 min, with each gas level interspersed by a 10 min recovery period in air. The order of presentation of hypoxic *versus* hypercapnic trials was randomized between individual animals. Cardiac responses to these conditions (heart rate, direction of contraction and velocity of contraction propagation) were determined during the final minute of the 10 min exposure to the control and experimental gases.

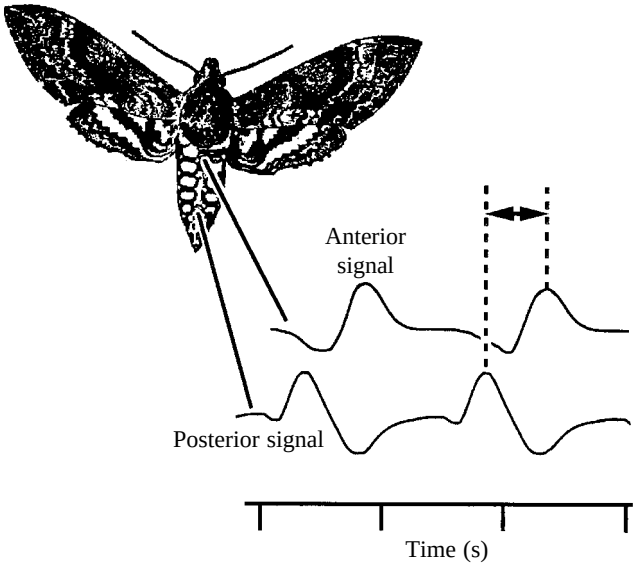

Fig. 1. Recording locations and resultant cardiac signals in *Manduca sexta*. Positive deflections occur when contraction of the dorsal vessel, which is propagated as a wave down the vessel, passes the recording electrode locations. The displacement between contractile events at the two recording sites (arrow), measured in seconds, in conjunction with the distance between electrode sites, permitted calculation of contraction propagation velocity, expressed in $cm\,s^{-1}$. Similar positioning of recording electrodes was used in larvae and pupae (not shown).

Temperature change

The effects of body temperature on cardiac performance were evaluated at three ambient levels (10, 20 and 30 °C) generated by cooling or warming the test flask in a temperature-controlled water bath. Test temperatures, confirmed by a thermistor probe placed next to the animal, were presented in a standard sequence (20, 10, 20, 30, 20 °C), with each temperature being maintained for 0.5 h. Only cardiac responses to changes in body temperature recorded after 10 min at the test temperature were analyzed. All animals appeared quiescent during these measurements, suggesting that no contribution of exercise-induced muscle contraction altered or adjusted body temperature to levels different from ambient.

Forced activity

Cardiac responses to forced activity were recorded for each developmental stage. Following a 10 min period of control measurements in resting, undisturbed animals, forced activity was induced for 2 min by continuous prodding with a blunt probe introduced through the mouth of the flask. Heart performance was measured continuously for 20 min from the onset of forced activity, with cardiac data plotted for a control, for 1 and 2 min of activity and for 3, 10 and 18 min after activity ceased.

Data analysis

Descriptive statistics of heart rate and velocity of contraction propagation were calculated for individuals of each stage and experimental condition. Comparisons of these mean cardiac values at rest as related to developmental stage were performed using one-way analysis of variance (ANOVA), followed by Student–Neuman–Keuls (SNK) *post-hoc* tests. Mean values of cardiac responses to sequential experimental treatments (O_2 and CO_2 levels, temperature and activity) were compared with control levels and each other using a repeated-measures one-way ANOVA, with subsequent SNK tests when significance between means was indicated. The level for significance was

set at $P<0.05$. Descriptive values reported in the text are means ± S.E.M.

Results

Heart rate patterns by developmental stage

The pattern of contraction of the dorsal abdominal vessel of *M. sexta* and its response to environmental perturbation appeared to change radically with metamorphic stage. The basic contractile rhythm, which we refer to as heart contraction, progressed from a relatively simple uniform pattern in the larval stage, to a highly complex and stereotypic pattern in the adult (Fig. 2). Heart rates of larvae averaged 34.8±1.16 beats min^{-1} (N=21) (Figs 2, 3); this rate was not significantly affected by body size, despite the large variation in the size and age of the larvae tested (Fig. 4; regression statistics, r^2=0.25, P>0.05; N=21). All heart contractions in resting larvae proceeded in a posterior to anterior direction.

The heart rate pattern in pupal *M. sexta* was non-uniform and displayed a high degree of rate variability (Fig. 2). Intervals of regular heart beats were frequently and irregularly interrupted by periods of cardiac arrests. The duration of cardiac arrests was also highly variable, ranging from a few seconds to over 20 min. No discernible rhythm of heart contraction emerged either within or among the pupae tested. When heart contractions continued for more

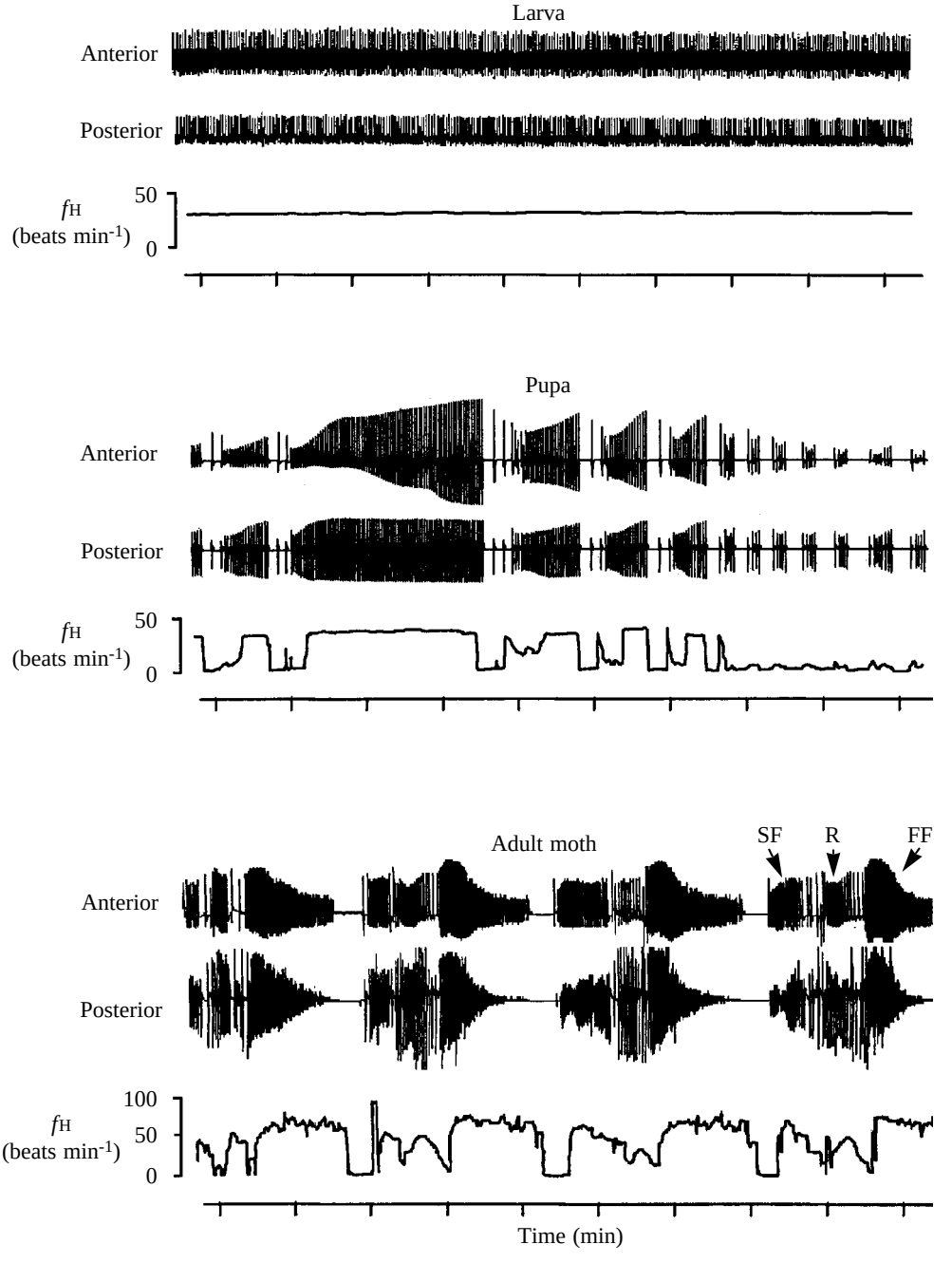

Fig. 2. Typical patterns of heart contraction recorded in resting unrestrained larval, pupal and adult *Manduca sexta* at 20 °C. Actual recordings from both anterior and posterior recording sites, as well as instantaneous heart rate f_H, are shown. Note the distinct and highly characteristic differences between developmental stages. Adult moths show periods of cardiac pausing as well as three distinct types of contraction: 'fast forward' (FF), 'slow forward' (SF) and 'reversals' (R). See text for further details.

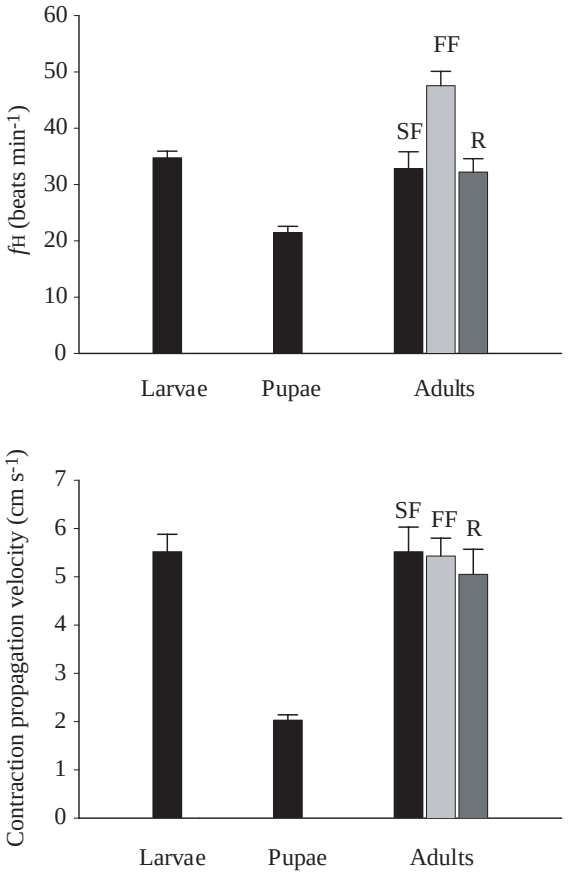

Fig. 3. Heart rate f_H and contraction propagation velocity as a function of developmental stage in larval, pupal and adult *Manduca sexta* at 20 °C. Mean values ± 1 S.E.M. are provided. *N* for larval, pupal and adult stages was 21, 7 and 9, respectively. Separate values are provided for the three distinctive 'slow forward' (SF), 'fast forward' (FF) and 'reversal' (R) patterns of adults.

than a few beats in a posterior-to-anterior direction, the heart rate was exceptionally constant (Fig. 2). Pupal heart rates during these uniform periods averaged 21.5±1.09 beats min^{-1} (*N*=7), a rate that was significantly lower than that of larvae and adults (Fig. 3; ANOVA, *P*=0.001). Heart rates were relatively constant over pupal body masses from 2 to 6 g (Fig. 4). Oscillations in the direction of the heart contraction ('reversals') were common but unpredictable.

Adult *M. sexta* displayed a complex but predictable pattern of heart contraction (Fig. 2). Heart contraction of resting adult moths consisted of a uniform sequence of three phases: a slow posterior-to-anterior phase (slow forward, SF), a slow anterior-to-posterior phase that crescendoed in speed (reversal, R), and a longer but faster posterior-to-anterior phase (fast forward, FF) that terminated abruptly in a cardiac arrest of variable length (Fig. 2). The rates of the SF and R phases were statistically similar at 32.8±3.0 and 32.2±2.4 beats min^{-1}, respectively, but were slower than that of the FF phase (47.6±2.6 beats min^{-1}, ANOVA, *P*=0.001, *N*=9). This complex pattern was evident in every resting adult tested, regardless of age or size. Despite the small variation in body size of adult moths, the average FF rates of individual moths varied between 40 and 60 beats min^{-1} (Fig. 4).

Contraction propagation velocity in larvae and pupae averaged 5.52±0.36 (*N*=21) and 2.03±0.11 cm s^{-1} (*N*=7), respectively (Fig. 3). That of the SF, R and FF phases of the adults averaged 5.52±0.51, 5.05±0.52 and 5.43±0.37 cm s^{-1} (*N*=9), respectively. The only contraction propagation velocity that differed significantly across development stage or phase was that of the pupae (ANOVA, *P*=0.001).

Cardiac responses to O_2 and CO_2

The heart rates of larvae were significantly affected by O_2 level (Fig. 5), but only at the level of anoxia (0 % O_2; ANOVA, *P*<0.001). The reduction in heart rates at 0 % O_2 was effected by a significant decrease in the contraction propagation velocity (ANOVA, *P*<0.004), but not by changes in the inter-beat (diastolic) interval (ANOVA, *P*>0.05). No reversals of heart contraction were seen in larvae during the O_2 series tests. Similar reductions in heart rates occurred in the SF and FF phases of adults, with only 0 % O_2 having a significant effect (ANOVA, both both *P*<0.001); no significant changes were observed in the heart rate during reversals (ANOVA, *P*=0.27). The contraction propagation velocity of adult hearts, irrespective of phase (SF, R or FF), was unaffected by the level of O_2 (ANOVA, all *P*>0.9).

Hypercapnic exposure in larvae caused a significant reduction in heart rate, but only at the highest level (24 %) of exposure (Fig. 6; ANOVA, *P*=0.007). The change in heart

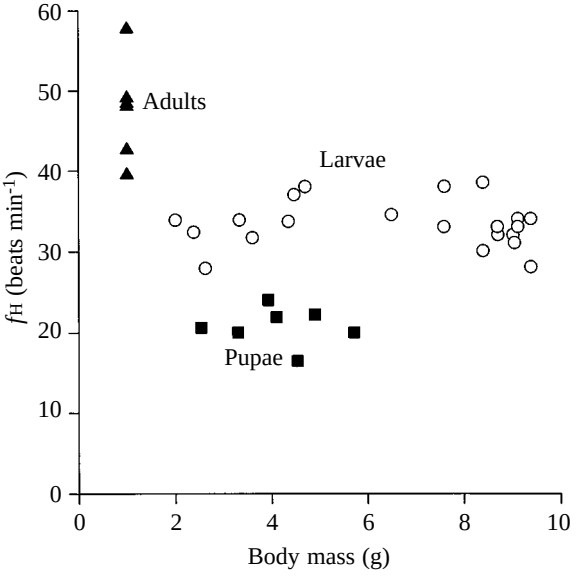

Fig. 4. Heart rate f_H as a function of body mass in larval (circles), pupal (squares) and adult (triangles) *Manduca sexta* at 20 °C. *N* for larval, pupal and adult stages was 21, 7 and 6, respectively. For adults, f_H during fast forward periods are plotted.

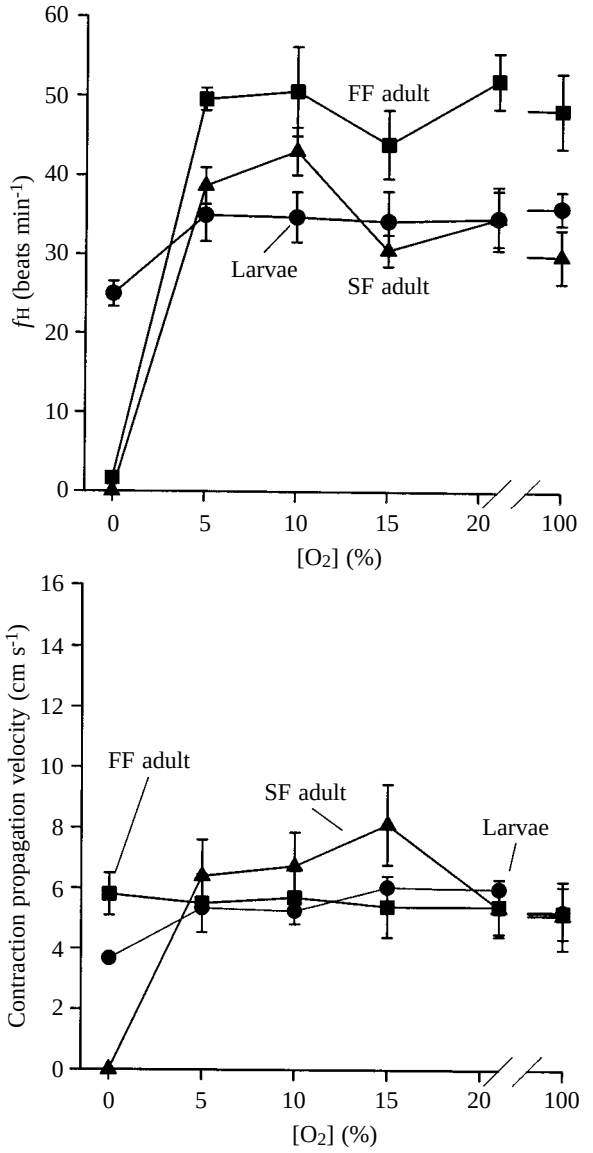

Fig. 5. Heart rate f_H and contraction propagation velocity as a function of ambient oxygen concentration in resting, unrestrained larval and adult *Manduca sexta* at 20 °C. Mean values ± 1 S.E.M. are provided. *N* for larvae and adults was 4 and 5, respectively. Separate values are provided for the 'slow forward' (SF) and 'fast forward' (FF) patterns of adults.

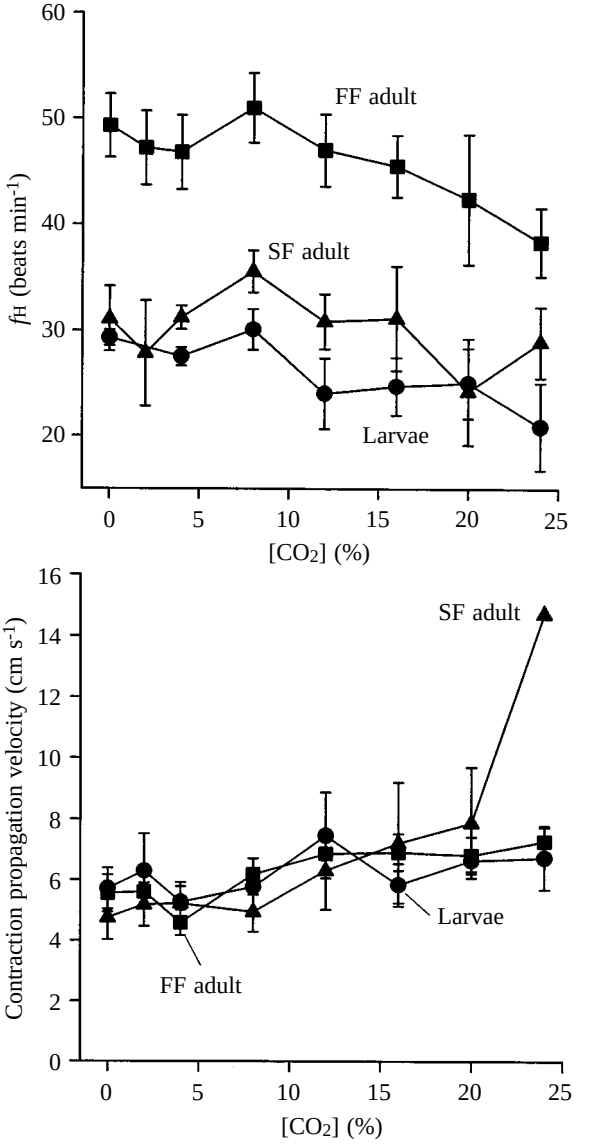

Fig. 6. Heart rate f_H and contraction propagation velocity as a function of ambient carbon dioxide concentration in resting, unrestrained larval and adult *Manduca sexta* at 20 °C. Mean values ± 1 S.E.M. are provided. *N*=5 for both larvae and adults. Separate values are provided for the adult 'slow forward' (SF) and 'fast forward' (FF) patterns.

rate during extreme hypercapnia was not effected by a decrease in the contraction propagation velocity (ANOVA, P=0.107). Heart rate reversals were observed in one of six larvae tested during hypercapnic trials. Increased levels of CO_2 caused gradual decreases in mean SF and mean FF heart rates in adult *M. sexta* (Fig. 6); a statistically significant reduction was found only in FF rates and only at the highest level (24 %) of CO_2 (ANOVA, P=0.0015). Reversal heart rates were unaffected by the level of CO_2. In contrast, increased CO_2 levels significantly increased contraction propagation velocity in both SF (P=0.025) and R phases (P=0.002) (ANOVA), but did not alter that in the FF phase (ANOVA, P=0.82). Measurements of cardiac responses of pupae to O_2 and CO_2 were not performed because of the chaotic resting pattern displayed by this stage and the consistent abdominal wriggling that several pupae displayed during initial O_2 and CO_2 trials.

Cardiac responses to temperature

As expected, the heart rates of all three developmental stages increased with increasing temperature (Fig. 7). For purposes of appropriate comparisons among stages, only the heart rates for the FF phase of the adult heart contractions were plotted and compared statistically. Within stages, heart

rates increased significantly between both 10 and 20 °C and between 20 and 30 °C for larvae and pupae (ANOVA, all $P<0.01$); only heart rate increases between 20 and 30 °C were significant in adults. Comparisons between stages and temperature indicate that adult FF heart rates differ from those of larvae and pupae at all temperatures tested (ANOVA, all $P<0.003$). Heart rate Q_{10} values for larvae, pupae and adults between 10 and 20 °C were 2.33, 3.14 and 1.61, respectively; corresponding Q_{10} values for these stages between 20 and 30 °C were 2.22, 2.03 and 2.29. Paired comparisons revealed no differences between Q_{10} within a stage, but the adult Q_{10} for heart rate differed from that of the other stages at the 10–20 °C step ($P<0.01$). Contraction propagation velocity changes reflected those of heart rate changes within stages (Fig. 7); differences were seen both between 10 and 20 °C and between 20 and 30 °C for larvae, and only between 20 and 30 °C for pupae and adults. However, comparisons among stages indicate no difference between larvae and adult contraction propagation velocity at any temperature.

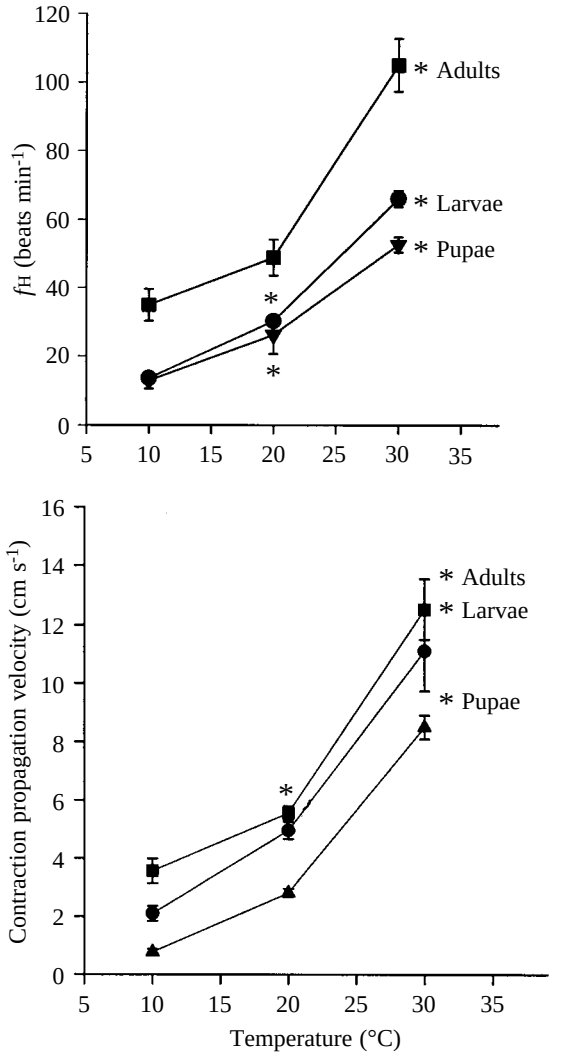

Fig. 7. Heart rate f_H and contraction propagation velocity as a function of ambient temperature in larval, pupal and adult *Manduca sexta*. Mean values ± 1 S.E.M. are provided. *N* for larval, pupal and adult stages was 6, 4 and 5, respectively. Contraction propagation velocity for adults represents fast forward (FF) values. An asterisk signifies a significant difference ($P<0.05$) in heart rate and contraction propagation velocity between adjacent test temperatures.

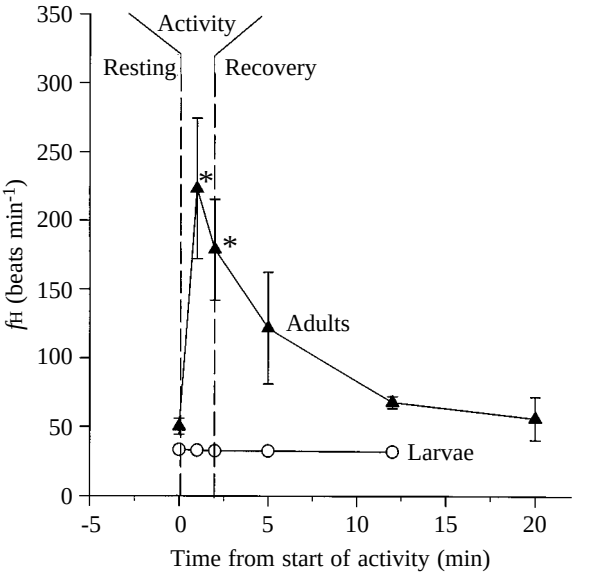

Fig. 8. Changes in heart rate f_H at rest, during intense activity stimulated by mechanical prodding and during recovery in unrestrained larval and adult *Manduca sexta* at 20 °C. Mean values ± 1 S.E.M. are provided. $N=5$ for both larval and adult stages. An asterisk signifies a significant difference ($P<0.05$) in heart rate from resting values.

Cardiac responses to forced activity

Adult *M. sexta* responded in a dramatic manner to forced activity by increasing their heart rate from 50.1±5.9 beats min^{-1} ($N=5$) during rest to 223.5±36.2 beats min^{-1} after 1 min of prodding (Fig. 8). Mean rates of heart contraction at 1 and 2 min of activity (period of forced activity) were significantly different from resting and recovery levels (ANOVA, $P=0.004$) and were characterized by uniform FF heart contraction during these elevated rates. In contrast, heart rates of larvae remained unchanged in response to forced activity (ANOVA, $P=0.096$, Fig. 8).

Discussion

The distinctive dorsal contractile blood vessel of insects has fascinated researchers for centuries yet, despite scores of published studies, our understanding of many aspects of circulatory control in insects, particularly intact insects, remains limited (Miller, 1985; McMahon et al., 1997b). Our contribution to this large database of circulatory control in insects involves studies of the developmental changes in the life stages of *M. sexta* and the information that emerges from these developmental comparisons.

General patterns

Patterns of cardiac contraction obtained in the present study from larval, pupal and adult *M. sexta* were as dissimilar as their morphological appearances and revealed a gradation from simple to complex (Fig. 2). Heart contractions of larval *M. sexta* were relatively monotonic, varied little between individuals in absolute rate and were devoid of cardiac pauses and impulse reversals (Figs 2–4). Despite a previous report that heart rates of larval moths (*Mamestra brassicae*) might decline with age (Queinnec and Campan, 1972), we observed an extremely repeatable rate of 35±1 beats min^{-1} (at 20 °C) for all *M. sexta* larvae tested, ranging in age from 10 to 21 days. This metronome-like pattern in larvae changed abruptly at metamorphosis to the pupal stage, which brought with it an unpredictable pattern that varied greatly between individuals, but was generally repeatedly interrupted by cardiac pauses and impulse reversals. Average heart rates of *M. sexta* pupae (21.5 beats min^{-1}) were the slowest of all stages and are the slowest rates reported for any intact lepidopteran pupae at this temperature (Miller, 1985). Metamorphosis of the *M. sexta* pupa to the adult moth revealed yet another pattern of heart contraction, best described as a repeating series of three distinct phases (SF, R and FF; see Fig. 2). While complex patterns of heart contraction have been reported for numerous species of adult moth (see Wasserthal, 1976; Miller, 1985; McMahon et al., 1997b) that include propagation reversals, our study represents the first report of a pattern of repeatable, well-defined phases. Such a complex and repeatable pattern may exist in other moth species, but may be displayed only when the individuals are completely undisturbed, as in our study. The basis for these distinctive, ontogenetic cardiac patterns is not clear, but they are undoubtedly related to developmental differences in both morphology and life-style. Larvae are anatomically 'homogeneous' compared with other stages, with no distinct head, thorax and abdominal region (or wings) that might require selective perfusion or drainage. Moreover, the exaggerated body movements during locomotion may also enhance the movement of hemolymph throughout the larval body.

The far more complex pattern of heart activity seen in pupae might have been predicted on the basis of the dramatic changes in internal morphology that are occurring during this stage. Simultaneous degradation and synthesis of tissues throughout the body may expose the heart to numerous peptides or neurohormones that affect cardiac activity. Moreover, this thorough restructuring may require a complex pattern of convective redistribution of synthetic raw materials. The very long and numerous cardiac pauses in this stage might also relate to histolysis of the cardiac tissue itself, as larval cardiac tissue is being replaced by the hemocytes that form the adult heart (Miller, 1979, 1985). The development of body regions and the decreased hemolymph volume (required for future lightweight flight) undoubtedly change the peripheral resistance of outflow vessels as well as venous return.

In adult moths, there are abundant reasons to expect the complex and repetitive pattern of cardiac activity that we observed. Typically, hemolymph is actively pumped from the abdomen to the thorax, head and wings through the anterograde contraction of the dorsal vessel and the contraction of the abdomen; return of hemolymph to the abdomen occurs ventrally through the perineural sinus, possibly during abdominal expansion and cardiac pauses (Wasserthal, 1981). Wasserthal (1976, 1981) has described the complexity of hemolymph movement in this species, and Heinrich (1971, 1981) has described the thermoregulatory capabilities of *M. sexta* and other species. These authors provide a convincing rationale for a well-regulated system of hemolymph movement between the thorax, wings and abdomen, not only passively associated with abdominal ventilatory movements, but also for the selective heating of body regions for the purposes of flight. Why the heart of pupal and adult *M. sexta* displays two different phases of anterograde beating, separated by retrograde beating, is unknown. From a hemodynamic perspective, changes in heart rate could result either from a change in volume flow coming to the heart (venous return) or from a change in peripheral resistance of the outflow vessels, or both. In the case of venous return, the short period of impulse reversal (R) may result in an accumulation of hemolymph in the abdomen (retrograde flow), stimulating the FF (anterograde flow) phase. Changes in the phase of anterograde flow (SF *versus* FF) may also be necessary to perfuse different vascular beds (Wasserthal, 1976; Miller, 1985).

As recognized in previous studies (see Wasserthal, 1976), impulse reversals in the cardiac pattern first appear in the pupal stages, and they become an integral part of the adult cardiac pattern. Contrary to earlier studies that viewed reversals as 'stress reactions' or 'disturbances of heart automatism' (McCann, 1970; Tenny, 1953), our study on undisturbed intact *M. sexta* supports Wasserthal's (1976) view that reversals are not only common and normal, but an essential part of the cardiac cycle, particularly in the adult developmental stages. Changes in heart rate (SF and FF) and direction (R) for the purpose of redistributing hemolymph in the older stages may be pivotal, because we recorded no major changes in the velocity of dorsal vessel contraction (contraction propagation). The propagation wave traveled down the vessel at nearly uniform rates (approximately 5 cm s^{-1}) in larval *M. sexta* and in all three phases of *M. sexta* adults (Fig. 3). This observation indicates that, despite the enormous changes that occur during metamorphosis, the fundamental cell–cell communication (excitation and contraction) within the dorsal vessel is maintained between larvae and adults. Why contraction propagation velocity is much lower in pupae is unknown; it may represent a temporary state due to cardiac histolysis. Our data showing the constant contraction propagation velocity in the three cardiac phases of adults are consistent with those of McCann (1964), who reports that the impulse conduction passes with equal facility and speed in both anterograde and retrograde directions in moth myocardium.

Effects of O_2, CO_2, temperature and forced activity

Pertubations to physiological systems often reveal the limits

and capacities of those systems, so we invoked changes in environmental P_{O_2}, P_{CO_2} and temperature as well as stimulated activity in *M. sexta* to attempt to tease apart the stage-specific differences in cardiac performance. Reduction of ambient O_2 and elevation of ambient CO_2 levels within ranges that might be encountered in natural environments had relatively little effect on heart rates and contraction propagation velocity in either larval or adult *M. sexta* (Figs 5, 6). Only total anoxia (0 % O_2) and CO_2 grossly elevated to 24 % caused significant reductions in heart rate. The responses to anoxia or near-anesthetic levels of hypercapnia probably represent direct ischemia and narcosis of the cardiac tissue. A variety of invertebrates respond with heart rate changes to much milder levels of environmental hypoxia or hypercapnia through receptor-mediated pathways or secondarily through ventilatory changes (McMahon et al., 1997b). However, in insects, the tracheal system, not the circulatory system, is responsible for O_2 and CO_2 transport. Thus, one might anticipate that, in response to hypoxic or hypercapnic exposure, the major rate and volume changes might occur in tracheal ventilation rather than in hemolymph pumping. In this regard, it is interesting to note that, while pupae, like larvae and adults, showed little cardiac response to hypoxia and hyperoxia, they did respond behaviorally to these gases by invariably and vigorously wriggling their abdomen. Such movements may have selective advantage in pupae, since wriggling in their subterranean refuge may be instrumental in reducing gas boundary layers forming against the pupa's body wall and perhaps even in ventilating their underground cavity.

Despite the seminal role of *M. sexta* in demonstrating the importance of the circulation in invertebrate heterothermic thermoregulation (Heinrich, 1971, 1981), we know of no previous measurements of cardiac performance across both developmental stage and temperature in *M. sexta*. While heart rates differed between stages, Q_{10} for heart rate and contraction propagation velocity were extremely similar in all stages (Fig. 7). Q_{10} values for heart rate in *M. sexta* are generally similar to those in other invertebrates (see McMahon et al., 1997b) and resemble the values of 2.2 reported for the dipteran fly (*Corethra plumicornis*) between 14 and 24 °C (Lagerspetz and Perttunen, 1962). Further, all stages displayed relatively muted changes in heart performance between 10 and 20 °C compared with the larger increases observed between 20 and 30 °C. This elevation in cardiac response at higher temperatures may represent a circulatory preparation for activity and flight, which only occurs when the adult moth has pre-warmed its flight muscles to 30–40 °C (Heinrich, 1971, 1981). The similarity in cardiac responses to temperature change across development stages suggests that metabolic underpinnings for these thermal responses occur very early in development and are not greatly affected by metamorphic reconstruction.

If the fundamental cellular construction of the *M. sexta* heart is so similar across developmental stages, at least on the basis of its chronotropic responses to temperature, why have we observed such different heart rate patterns in larvae, pupae and moths? The answer appears to be that the addition of modulating elements to the circulatory system of pupae and adults allows them to generate more complex patterns than the metronome-like pattern of larvae. One of these modulating elements could be the development or activation of an anterior pacemaker in pupae and adults (and perhaps the oldest larval instars) that enables retrograde contraction of the dorsal vessel. Retrograde contraction is essential for the 'wing spreading' of adults, but must be activated by neurohormones (cardio-acceleratory peptides), a second and important developmental element that modulates the cardiac pattern in numerous insect species (Miller, 1985; Tublitz, 1989; Tublitz et al., 1992; McMahon et al., 1997b). An apparent absence of neural or neurohormonal influence on the heart of larval *M. sexta* is perhaps best demonstrated in the larva's complete lack of response to forced activity (Fig. 8), contrasted with the rapid and transient elevation of heart rates in adult moths. Not surprisingly, the heart rate of adult bees similarly increases during activity (Schaub et al., 1991). In essence, larvae appear to possess a dorsal contractile vessel that has the basic *M. sexta* endogenous rhythm, propagation velocity and anterograde direction of contraction, but lacks the neural projections, neurohomonal release and functional second pacemaker that are required for the complex cardiac pattern exhibited by adults.

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