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Dynamic mechanical oscillations during metamorphosis of the monarch butterfly

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The mechanical oscillation of the heart is fundamental during insect metamorphosis, but it is unclear how morphological changes affect its mechanical dynamics. Here, the micromechanical heartbeat with the monarch chrysalis (*Danaus plexippus*) during metamorphosis is compared with the structural changes observed through *in vivo* magnetic resonance imaging (MRI). We employ a novel ultra-sensitive detection approach, optical beam deflection, in order to measure the microscale motions of the pupae during the course of metamorphosis. We observed very distinct mechanical contractions occurring at regular intervals, which we ascribe to the mechanical function of the heart organ. Motion was observed to occur in approximately 15 min bursts of activity with frequencies in the 0.4–1.0 Hz range separated by periods of quiescence during the first 83 per cent of development. In the final stages, the beating was found to be uninterrupted until the adult monarch butterfly emerged. Distinct stages of development were characterized by changes in frequency, amplitude, mechanical quality factor and de/repolarization times of the mechanical pulsing. The MRI revealed that the heart organ remains functionally intact throughout metamorphosis but undergoes morphological changes that are reflected in the mechanical oscillation.

Keywords: monarch butterfly; heart; mechanics; oscillation; development; metamorphosis

1. INTRODUCTION

Biochemical and mechanical oscillations as diverse as calcium signalling, the cell cycle, heartbeats and the circadian rhythm are all key control mechanisms for the viability of living organisms (Shroff *et al.* 1995; Goldbeter 1996; Domke *et al.* 1999; Pelling *et al.* 2004; Kruse & Julicher 2005; Vilfan & Frey 2005; McHale *et al.* 2006). The larva of the monarch butterfly (*Danaus plexippus*; a Lepidoptera) transforms into a jade green pupa (figure 1*a*) that undergoes metamorphosis (lasting approx. 7–10 days) to become the adult (Oberhauser & Solensky 2004; figure 1*b*). Approximately 24 hours before the adult butterfly appears, the chrysalis becomes transparent and the adult can be seen (Oberhauser & Solensky 2004; figure 1*a*). In this study, we used an optical beam deflection (OBD) set-up (figure 1*c*) similar to an atomic force microscope (AFM; Binnig *et al.* 1986; Meyer & Amer 1988) to measure the surface motion of the pupa during metamorphosis. Although the AFM has been extensively employed to study the mechanics of single molecules and cells (Hinterdorfer & Dufrene 2006), it has rarely been employed to study whole organisms (McConney *et al.* 2007).

Using OBD sensing, we were able to measure periodic mechanical motion that we ascribed to the heart. The heart organ is generally considered to be the abdominal part of the dorsal vessel that runs along the axis of the body (Slama 2000) and consists of a series of pumps (Snodgrass 1935). The heart drives the haemolymph around the body in an open circulatory system and is known to exhibit heartbeat reversal, changes in mechanical pressure, consumption and release of O_2/CO_2 and fluctuations in temperature (Gerould 1929; Slama 2000, 2003; Smits *et al.* 2000; Tartes *et al.* 2000; Slama & Neven 2001; Slama & Farkas 2005). Based on the micromechanical phenomena observed in this study, we propose that the heart remains functionally intact even though massive structural development occurs during metamorphosis.

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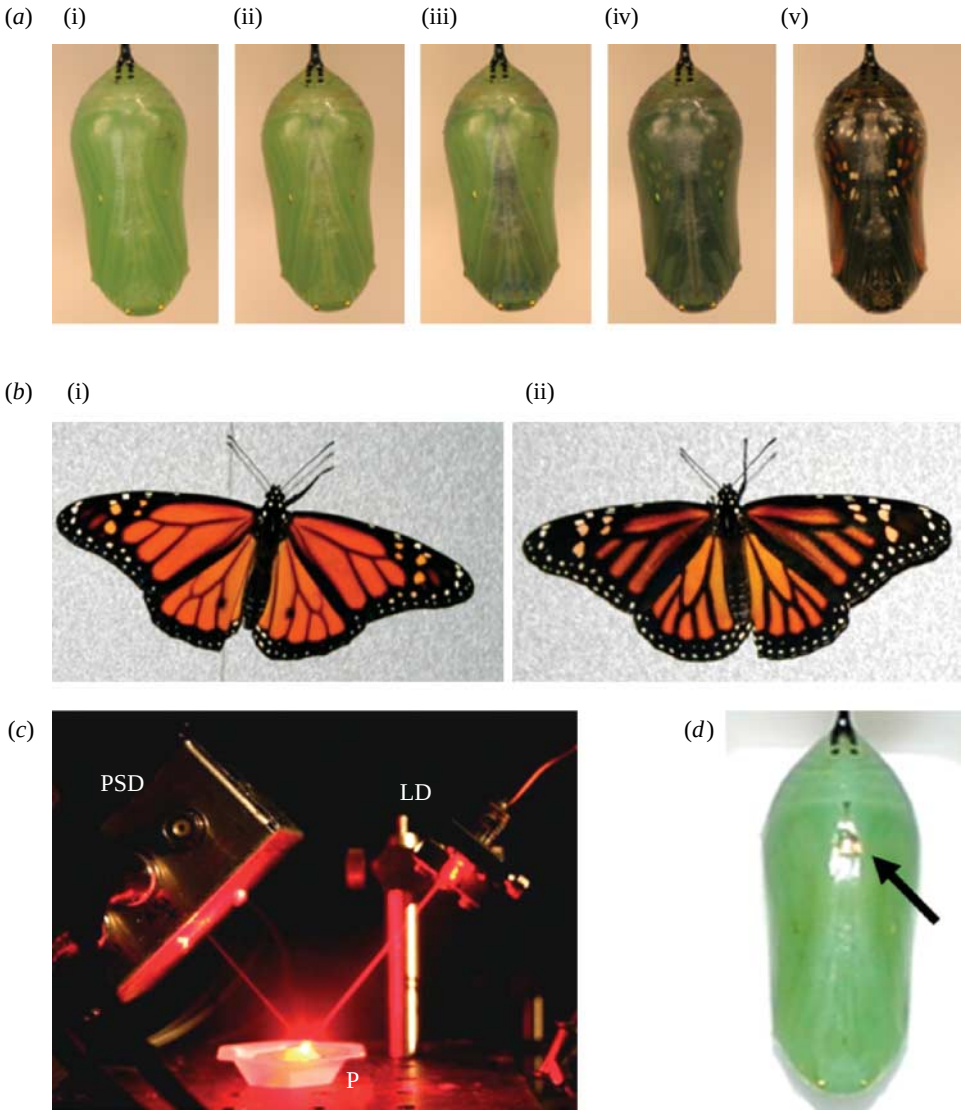

Figure 1. (*a*) The chrysalis is initially green during development (D) and approximately 2×1 cm ((i) 0–50%D, (ii) 70%D, (iii) 80%D, (iv) 90%D, (v) 100%D). The end stages of development are marked by the chrysalis losing its colour revealing (*b*) the adult butterfly that will emerge approximately 24 hours after this stage ((i) male, (ii) female). (*c*) The OBD motion detection system employs a laser diode (LD), focused onto a micromirror glued on the pupa (P) and reflected into a position-sensitive detector (PSD). (*d*) A small silicon nitride micromirror with approximately 500 μm^2 of gold coating is attached to the ventral side over the heart.

2. MATERIAL AND METHODS

2.1. Monarch chrysalides

Danaus plexippus chrysalides (pupae) were from a second-generation colony in Lancaster, PA. The colony was maintained during the summer of 2004 in a 12× 12 ft breeding tent covered on four sides by mosquito netting. The monarch butterfly colony was started from two dozen fresh healthy adults obtained from the Florida butterfly farm (Shady Oak Nursery in Brooker, FL). Adult butterflies were immediately released into the breeding tent where they were allowed to mate and lay eggs on the leaves of a potted milkweed plant kept in the breeding tent. Larvae hatched from the eggs and the developed caterpillars transformed into pupae. Hardened chrysalides were gently removed from their perch and used for heartbeat measurements. All pupae appeared to be healthy as evidenced by the fact that healthy adult butterflies emerged from each one.

2.2. OBD set-up

The motions of the pupa were observed using OBD (Meyer & Amer 1988). Silicon nitride micromirrors (1 mm^2) coated with approximately 500 μm^2 of gold were glued (Elmer's all purpose, non-toxic glue) onto the surface of the pupae by applying a small amount of glue to the backside of the mirror with a microcapillary tube (figure 1). A second small drop of glue was used to affix the pupae to the sample stage. The gold-coated micromirrors were found to significantly increase the signal-to-noise ratio of the OBD method. The experiments were conducted using an OBD system geometry similar to that used in atomic force microscopy (Binnig *et al.* 1986; Meyer & Amer 1988). The light source was a 5 mW laser diode (LD) at 650 nm (US Lasers, Inc.), and the detector was a quadrant photodiode (QP50-6SD, Pacific Silicon Sensor, Inc.). The signals from the quadrant photodiode were recorded using a data acquisition card and LabVIEW software at a

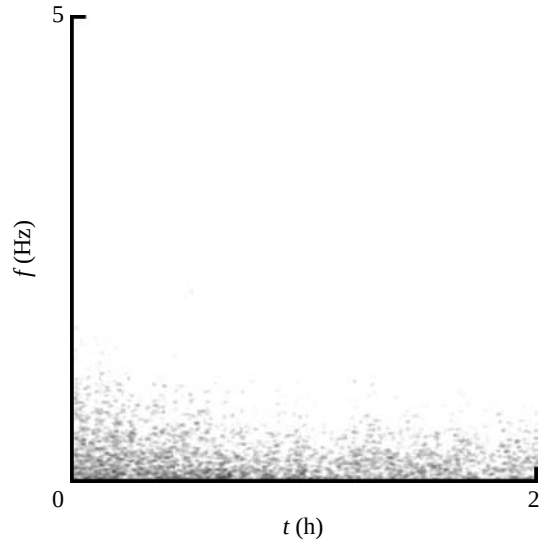

Figure 2. Spectrogram recorded on a dead pupa. The spectrogram displays the observed frequencies in the motion as a function of time. The spectrum is structureless and no periodic motions are observed.

sampling rate of 100 Hz. Data were high-pass filtered at 0.1 Hz and analysed with SpectraPRO v. 3.32 software (Cetacean Research Technology). Six healthy pupae were measured in this study and the results presented are representatives of the average spectral properties of the observed micromechanical motion.

The mechanical motion of the pupa was recorded with the OBD set-up after aligning a laser beam on a micromirror attached to its surface (figure 1*d*). The beam was aligned parallel to the long axis of the pupa. Care was taken to minimize disturbances by sound or light, and the measurements were conducted in a dark, acoustically isolated, temperature controlled room (22.5 ± 0.5°C). The spectral characteristics of recorded motion were analysed using spectrograms in which the beat frequency and amplitude are plotted as a function of time on a contour plot. A baseline was recorded using a dead pupa, which revealed a structureless background with a noise level of 0.5 ± 0.1 nm root-mean-squared (r.m.s.) as seen in the spectrogram (figure 2). By contrast, the live pupa displayed characteristic forms of periodic micromechanical motion. The motion observed is almost completely due to the vertical movements of the surface of the chrysalis and not the angular motions associated with a travelling wave. This was confirmed by rotating a chrysalis by 90° (to achieve a position in which the long axis of the pupa was perpendicular to the laser beam direction) during mechanical beating. If the observed oscillations were due to angular motion rather than translational motion, the signal we observed in the top–bottom channel would have shifted to the left–right channel on rotation of the pupae. Our observations determined that the angular motion is negligible because, in both configurations, the oscillatory signal was present only in the top–bottom channel of the position-sensitive detector (PSD). Therefore, due to the large size of the micromirror (relative to the heart) and placement directly over the heart, the angular motion was determined to be negligible in comparison with the amplitudes of the vertical beating.

2.3. Magnetic resonance imaging

Magnetic resonance imaging (MRI) was carried out with a 9 T magnetic field with water proton density weighted and water diffusion weighted scanning. The MRI imaging allowed us to determine the location of the heart enabling us to correctly position the mirror. The MRI was located at the Center for In Vivo Microscopy (Duke University Medical Center's Department of Radiology) and has been described previously (Stringer 2000).

3. RESULTS AND DISCUSSION

3.1. Mechanical dynamics define distinct stages of development

Pupae from different breeding periods over the course of the 2004 season were measured and displayed highly reproducible traits. Pupae from the end of the season did not have a high rate of survival and often displayed erratic and inconsistent beating properties if they survived. This is presumably owing to the high amount of inbreeding and genetic mutation, which occurs during this time. Trends were observed in the data when performing the analysis in terms of per cent development (%D). This allowed the data to be normalized by the total number of development days that was necessary due to the variation in each chrysalis (7–10 days). Data collection from one pupa made during a 2 hour interval at the same time each day are presented here and are considered to be representative of all healthy pupae that were measured in this study ($n=6$). The recordings revealed periods of beating activity lasting 15 ± 3 min interspersed with periods of quiescence (figure 3). We define these periods of activity as a beating burst (BB) and require that the motion be sustained for approximately 15 min and have a r.m.s. amplitude greater than 10 per cent of the r.m.s. amplitude during quiescence. The number of BBs per 2 hour period was also found to correlate with the increasing %D. Distinct development stages were observed which correlated to spectral characteristics such as the maximum beating frequency (f), the r.m.s. amplitude (A) and the number of BB (table 1).

3.2. Analysis of beating characteristics as a function of development

The 0–50%D stage (figure 3*a*) displayed one BB with a maximum average r.m.s. amplitude of 2.67 ± 0.15 µm (figure 3*a*). The spectrogram displays a maximum principal frequency at 0.52 ± 0.03 Hz, which has several overtones (figure 3*b*). The periodic motions (figure 3*c*) are characteristic of a heartbeat (Gerould 1929; Slama 2000, 2003; Smits *et al.* 2000; Tartes *et al.* 2000; Slama & Neven 2001; Slama & Farkas 2005) and their frequency was observed to increase and decrease in amplitude during the BB (figure 3*d*). The next stages of development 50–75%D and 75–83%D (figure 3*e*,*f*) display very

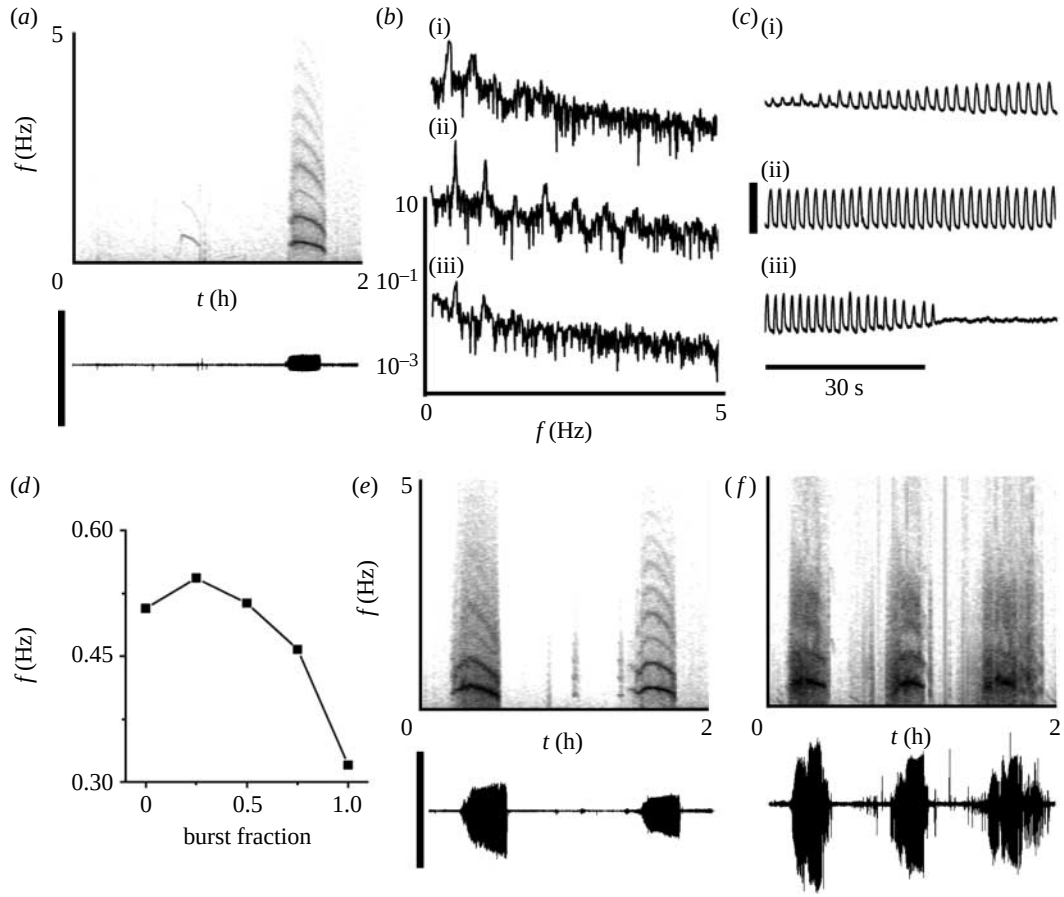

Figure 3. Characterization of beating burst (BB) spectral characteristics. The 0–50%D stage is characterized by one BB per 2 hour period. (*a*) The spectrogram reveals several overtones (approx. 7) above the principal frequency that reaches a maximum of 0.52 ± 0.03 Hz (amplitude scale is 10 μm and applies to all spectrograms). The maximum r.m.s. amplitude during the BB is 2.66 ± 0.15 μm as seen in the time-series data below (scale bar, 50 μm). (*b*) Fast Fourier Transform (FFT) slices measured at five points along the BB corresponding to specific burst fractions (bf) between 0 and 1.0. FFTs at (i) bf = 1.0, (ii) bf = 0.5 and (iii) bf = 0 are shown (offset for clarity) and the FFT slice at bf 0.5 clearly displays several overtones. (Note. The amplitude axis is logarithmic in μm.) (*c*) Periodic heartbeat motions corresponding to the FFTs in (*b*) are shown, which reveal the start, middle and end of the BB ((i) bf = 0, (ii) bf = 0.5, and (iii) bf = 1.0; scale bar, 10 μm). (*d*) The principal frequency increased and decreased during each BB observed in every stage. (*e*) The 50–75%D stage displayed similar spectral characteristics to the 0–50%D stage except that there were two BBs per 2 hour (scale bar, 50 μm). (*f*) The 75–83%D displayed three BBs per 2 hour period but displayed fewer overtones and sharp BBs in the spectrogram due to the appearance of non-beating motions (scale bar, 50 μm).

similar characteristics to the 0–50%D stage except they have two and three BBs per 2 hour period, respectively. Congruently, they also display similar trends in the average maximum principal frequency and r.m.s. amplitude during the BB but with larger magnitudes (figure 4).

Pupae displayed a drastic change in beating patterns during the 83–92%D stage as can be seen in the spectrogram (figure 5*a*). Beating motion became erratic in both duration and frequency and distinct BBs were no longer observed. Sudden occasional large-scale motions resulting from spontaneous twitching and movement would sometimes reach as much as 400 μm in amplitude. At this stage of development, the surface of the chrysalis became clear, marking the end of the pupal stage revealing the orange, black and white colouring of the adult wings (Oberhauser & Solensky 2004; figure 1*c*).

The last stage of development (92–100%D) was marked by consistent beating at approximately 0.95 ± 0.02 Hz (figure 5*b*). Beating was almost completely uninterrupted during this time and was consistent in the 8–12 hours prior to the emergence of the adult.

Table 1. Specific stages of development of the pupae are marked by distinct frequency and amplitude heartbeat properties.

stage (%D)	BB	max principal frequency (Hz)	r.m.s. amplitude (μm)
0–50	1	0.52 ± 0.03	2.66 ± 0.15
50–75	2	0.54 ± 0.02	7.56 ± 2.95
75–83	3	0.64 ± 0.04	13.31 ± 1.07
83–92	erratic beating	0.72 ± 0.03	17.33 ± 0.23
92–100	constant beating	0.95 ± 0.02	26.43 ± 0.87

When the adult emerged, a large off-scale signal was observed and the measurement was stopped. Healthy male and female butterflies (figure 1*c*) were raised during the experiment and were released; however, no identifiable differences in beating motion between males and females were observed in this study. An exponential growth in the maximum principal beat frequency and r.m.s. amplitude was observed over the course of metamorphosis in all specimens (figure 5*c*,*d*).

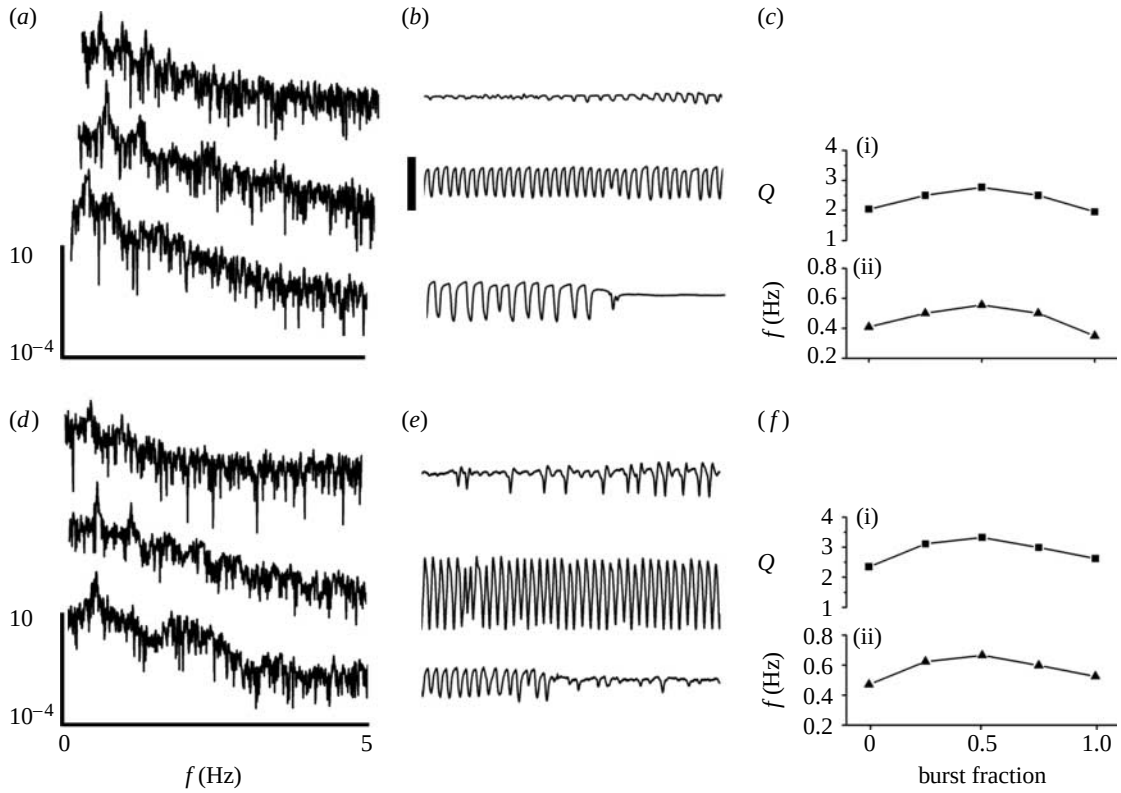

Figure 4. 50–75%D FFT slices from the first BB in the spectrogram (figure 3*e*) at (*a*) different bf and (*b*) corresponding time series are very similar to the 0–50%D stage with a similar average maximum frequency (0.54 ± 0.02 Hz) and slightly larger average maximum r.m.s. amplitude (7.56 ± 2.95 μm). (*c*) The BB at this stage also displays the characteristic changes in (i) Q-factor and (ii) frequency during the BB. The 75–83%D stage displays three BBs per 2 hour period (figure 3*f*) but the number of well-defined overtones has decreased as seen in the FFT slices from (*d*) the first BB and (*e*) time series. The average maximum frequency (0.64 ± 0.04 Hz) and r.m.s. amplitude (13.31 ± 1.07 μm) during the burst have increased significantly and each FFT slice now displays more background spectral noise. This is correlated to more complex beating patterns during the BB as seen in the time series shown in (*e*). (*f*) Consistent with the previous stages, (i) characteristic change of Q versus bf and (ii) the frequency displays a characteristic time dependence during the BB.

The beating motion also tended to lack the high number of strong overtones in later stages when compared with BBs during the first 0–75%D. Taken together, these results suggest that the heart becomes a stronger and more efficient pump during metamorphosis.

3.3. Analysis of late-season pupae

Peculiar mechanical oscillations were detected in monarch pupae ($n=3$) of the migratory generation (figure 6). The mechanical oscillations in this group were characterized by little or no bursting behaviour or consistent changes in frequency and/or amplitude during development. Surviving migratory butterflies (only 3 out of 10 pupae survived in our laboratory) were in reproductive diapause and typically live approximately eight times longer than their reproductive cousins from earlier in the season (Tatar & Yin 2001). These late-season monarchs are larger and of the cohort migrating to the transverse Neovolcanic belt range in Mexico where they roost through the winter and in spring to reinitiate the northern cohorts. Tentatively, anatomical and physiological differences (Herman & Tatar 2001) in these late-season monarchs may be responsible for the peculiar mechanical oscillations detected. However, it is also possible that the atypical mechanical oscillations may result from inbreeding within the colony as it was observed that many of the butterflies were diseased and did not emerge from their pupae (approx. 70%, we maintained 10 pupae in the laboratory and measured three for the data presented here). These results reveal that reproducible beating patterns and changes in frequency and amplitude are a characteristic signature of healthy pupae that are likely to survive to adulthood.

3.4. Geometric shape parameters of beating motion

To estimate the geometric shape and kinetics of the beating behaviour, we measured the mechanical de/repolarization times (table 2) as described in a previous study (Domke *et al.* 1999) on cardiomyocytes (figure 7). The depolarization (rise) time is calculated from the inverse of the maximum slope ($\mathrm{d}h/\mathrm{d}t$) of the pulse normalized by its height (Δh) where $t_{\mathrm{dep}} = 1/[(\mathrm{d}h/\mathrm{d}t)/\Delta h]$; likewise the repolarization time (t_{rep}) is measured from the downward slope of the pulse. Therefore, if the ratio of the depolarization and repolarization times ($t_{\mathrm{dep}}/t_{\mathrm{rep}} = [\mathrm{d}h_{\mathrm{rep}}/\mathrm{d}t]/[\mathrm{d}h_{\mathrm{dep}}/\mathrm{d}t]$) is greater than 1, then the heart is physically contracting much faster than when it expands and vice versa for ratios less than 1. Therefore, the ratio provides a description of the kinetics of the mechanical pulsations as well as a

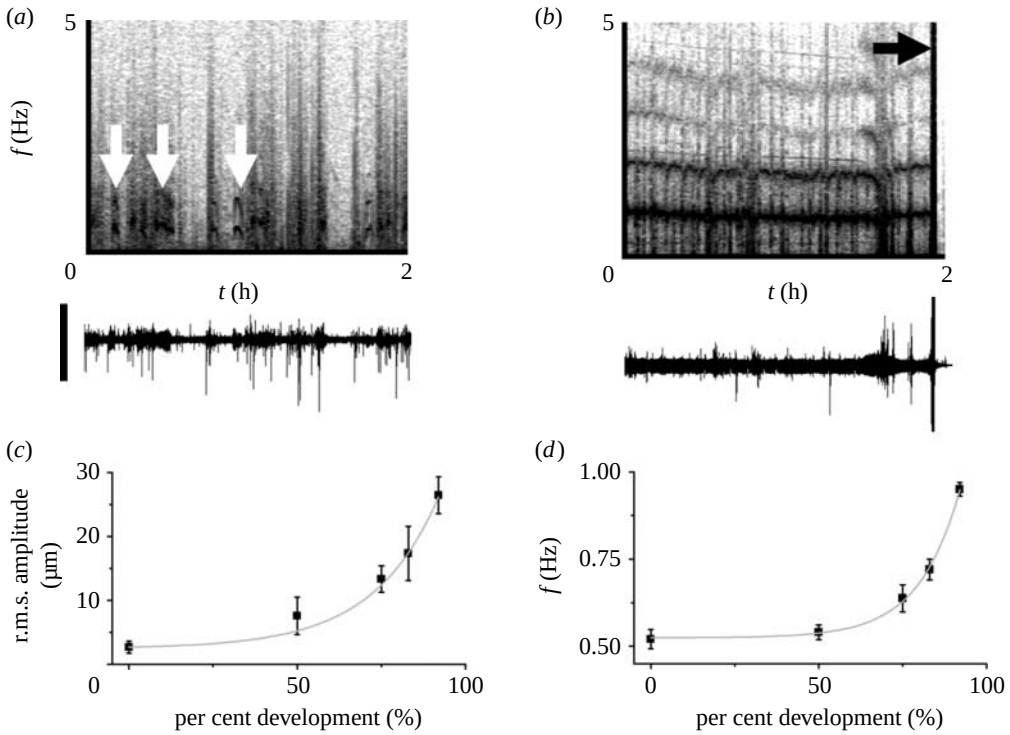

Figure 5. Characterization of the heartbeat during the 83–92 and 92–100%D stages. (*a*) The 83–92%D stage displays erratic beating at 0.72 ± 0.03 Hz (white arrows) with faint overtones. Large-scale motion reaching amplitudes of approximately 200 μm is observed (scale bar, 100 μm). (*b*) The final 2 hours of motion show constant and uninterrupted beating with a frequency of approximately 0.95 Hz. Emergence is marked by motion that goes off-scale (black arrow). The important spectral characteristics from multiple butterflies reveal a clear trend during metamorphosis (scale bar, 100 μm). Both (*c*) the r.m.s. amplitude and (*d*) the average frequency grow exponentially.

qualitative description of the pulse waveform. The t_{dep} and t_{rep} times were observed to display the trends during BBs (figure 7*a*) and specific to stages of %D (figure 7*b*). During a BB, the t_{dep}/t_{rep} ratios were observed to display linear trends dependent on %D. During the 0–50%D, the t_{dep}/t_{rep} ratio increased during a BB, decreased during the 50–75%D stage and became almost constant during the 75–83%D stage. The t_{dep}/t_{rep} ratios were also found to increase to a maximum of approximately 1.4 at 50–75%D stage and then decrease to approximately 0.8 at the 92–100%D stage, which was similar to the initial level (approx. 0.7). This reveals that a 0–50%D pulse has a quick rise followed by a long relaxation; however, during the 50–75%D stage, this trend is reversed and by the 92–100%D stage there is a recovery. Interestingly, the values obtained for t_{dep} and t_{rep} are approximately 10 times larger than those obtained on chicken embryo (Domke *et al.* 1999) and neonatal rat cardiomyocyte cells (Haupt *et al.* 2006; Pelling *et al.* 2007), indicating a difference in the functioning of the whole organ as opposed to the single cell.

3.5. MRI of developing pupae

MRI was employed to visualize the internal structures and organs of the pupae throughout metamorphosis (Stringer 2000; figure 8*a*). The MRI slices along each axis of the chrysalis (figure 8*a*) allow an analysis of internal development, which previously described several major organs (Stringer 2000). We chose an MRI plane by eye, which appeared to contain the heart organ. Owing to the fact that the structures are undergoing morphogenesis, this task was not trivial. However, by comparing the heart with other developing structures (abdomen and muscle features), we found that the heart typically ran through a single plane. In the data presented here, the heart appears in slice vii (figure 8*a*). Importantly, the data revealed that the heart stays intact throughout development and undergoes significant structural changes (figure 8*b*). Examining the heart organ in the 60, 80 and 100%D stages (100%D indicates a pupa that emerged on the same day as the MRI image), it is clear that the heart becomes approximately 52 per cent thinner and approximately 15 per cent longer during development.

Very little work has been done to characterize the development of the *D. plexippus* heart during metamorphosis, but the beating frequencies and BBs which we observe are highly consistent with other species (Gerould 1929; Slama 2000, 2003; ; Smits *et al.* 2000; Tartes *et al.* 2000; Slama & Neven 2001; Slama & Farkas 2005). The MRI reveals that the late-stage heart is composed of denser muscle, consistent with the observations in other insects (Gerould 1929). A higher rate of metabolism as the organism enters the late stages of development will require greater haemolymph flow and correlates with the structural changes in the heart muscle and the increased beating frequency and amplitude. The change in frequency and amplitude may be facilitated by the development of an extensive

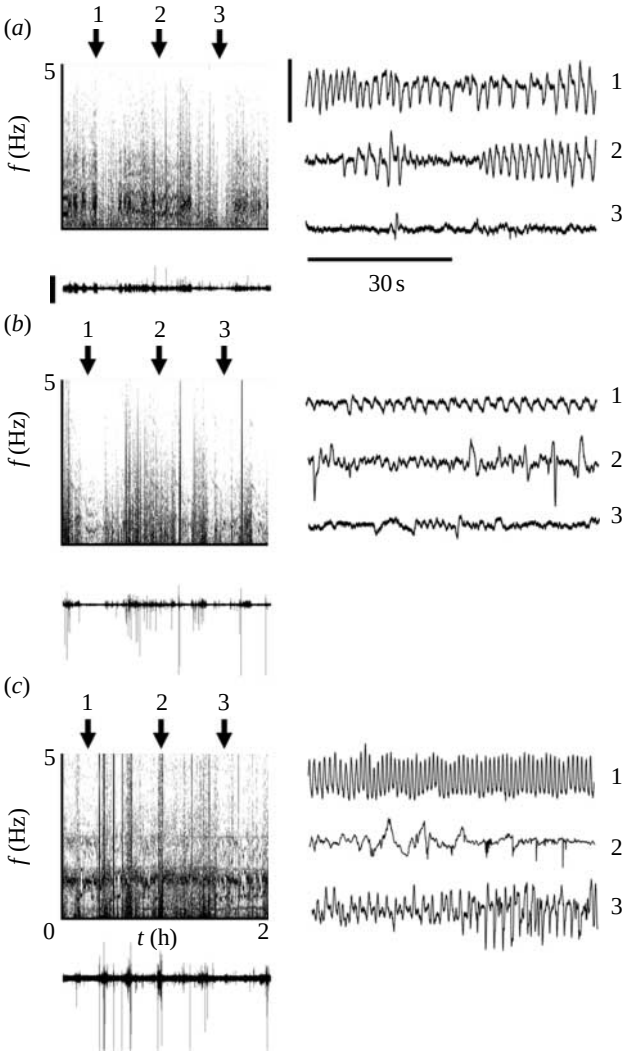

Figure 6. (*a–c*) Late-season migratory pupae often did not survive until emergence and typically displayed erratic beating patterns with no identifiable patterns or trends. These data were measured on one pupa. Data from (*a*) day 2, (*b*) day 4 and (*c*) day 6 are shown. The pupa was dead on the morning of day 7. A 2 hour FFT is shown with the corresponding time-series data below (scale bar, 100 µm and applies to all). Three 1 min time-series data (scale bar, 50 µm and applies to all) are shown for each day and correspond to the arrows in the FFT. Beating is still observed during the 6 days; however, the amplitude and frequency do not display any trends that are consistent with healthy pupae. The data reveal that pupae health and the likelihood of survival during metamorphosis can be determined by the examination of the mechanical pulsing behaviour during metamorphosis.

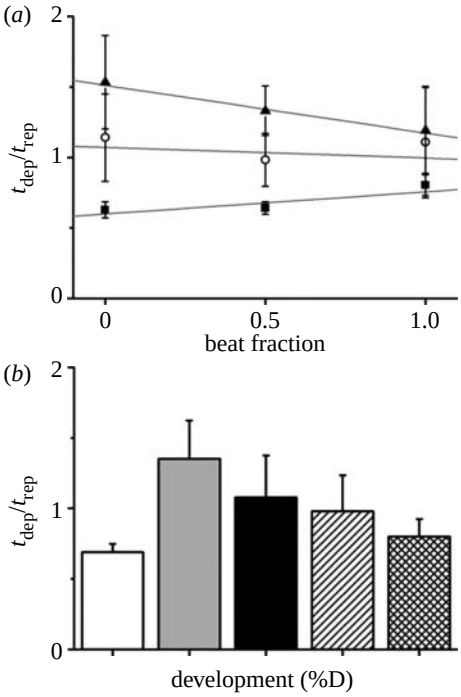

Figure 7. (*a*) The t_{dep}/t_{rep} ratio changes during BBs in the 0–50%D (squares), 50–75%D (triangles) and 75–83%D (circles) stages. (*b*) As well, over the course of metamorphosis, the t_{dep}/t_{rep} ratio (measured at bf=0.5) was observed to change (0–50%D (white bar), 50–75%D (grey bar), 75–83%D (black bar), 83–92%D (diagonal-line bar) and 92–100%D (crossed-line bar)).

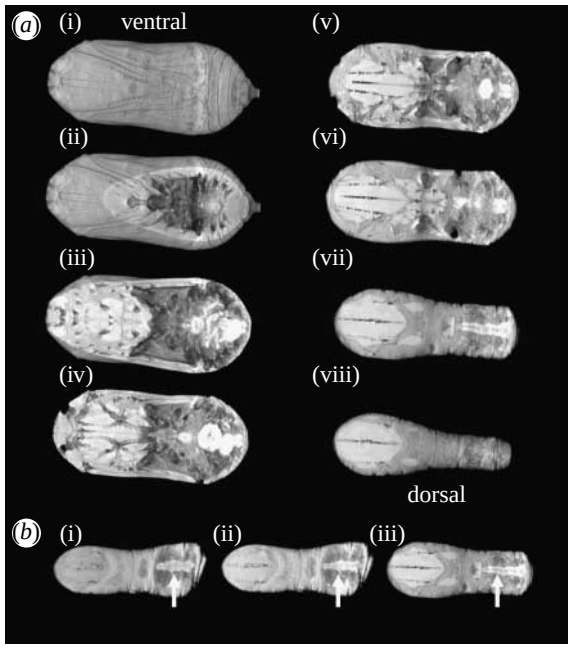

Figure 8. MRI of living pupae during development reveals heart development. (*a*) An example of the MRI slices in the ventral to dorsal direction ((i)–(viii)) of a pupa just before the adult emerged (100%D), revealing various internal organs such as the heart (slice vii). (*b*) The heart is shown during development; morphological changes occurring during the 80–100%D stages are clearly seen ((i) 60%D, (ii) 80%D, (iii) 100%D). The heart is approximately 52% thinner and approximately 15% longer in the 100%D stage than in the 60%D stage, and is much denser (brighter intensity in the 100%D image) consistent with the previous literature (Gerould 1929).

Table 2. Average depolarization time (t_{dep}), repolarization time (t_{rep}) and t_{dep}/t_{rep} ratio observed in this study. (Values are quoted as average ±s.d.)

stage (%D)	t_{dep} (s)	t_{rep} (s)	t_{dep}/t_{rep}
0–50	0.24±0.03	0.35±0.05	0.69±0.01
50–75	0.46±0.10	0.35±0.11	1.23±0.48
75–83	0.43±0.10	0.40±0.17	1.08±0.54
83–92	0.25±0.07	0.26±0.06	0.98±0.26
92–100	0.21±0.03	0.27±0.03	0.80±0.13

nervous system around the heart during metamorphosis, which has been observed in other insects (Gilbert & Frieden 1981).

4. CONCLUSIONS

Here, naturally occurring mechanical oscillations of the active heart can be measured using ultra-sensitive detection schemes on a whole organism. This novel application of a technique typically reserved for the detection of molecular or cellular motions at the nanometre scale (Radmacher *et al.* 1994; Shroff *et al.* 1995; Thomson *et al.* 1996; Domke *et al.* 1999; Szabo *et al.* 2002; Pelling *et al.* 2004) is clearly applicable to whole organisms on the centimetre scale. In the course of this research, OBD (Meyer & Amer 1988) and MRI (Stringer 2000) enabled the measurement of the mechanical development of the *D. plexippus* heart and the correlation to specific morphological trends during the progress of metamorphosis. We observed mechanical motion in approximately 15 min BBs with variable frequencies (0.4–1.0 Hz) separated by periods of quiescence. The number of BBs per 2 hour period allowed us to identify distinct stages of metamorphosis. Stages of development were characterized by changes in frequency, amplitude, mechanical quality factor and the geometric parameters of the contractions (de/repolarization times) of the mechanical pulsations. In the final stages of metamorphosis, the beating was found to be uninterrupted until the adult monarch butterfly emerged. The MRI revealed that the heart organ remains functionally intact throughout metamorphosis, but undergoes morphological changes that are reflected in the mechanical oscillation. OBD measurements do not appear to resolve the heartbeat reversal that is known to occur in other species (Slama 2000, 2003; Slama & Neven 2001; Slama & Farkas 2005); however, this does not preclude its existence. Results from pupae that did not survive metamorphosis reveal highly irregular mechanical beating behaviour throughout their development. These results show that the predictable mechanical beating observed during development is a characteristic marker of pupae that have a high likelihood of survival, eventually emerging as a fully formed adult monarch butterfly.

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