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Vibratory communication in the soil: pupal signals deter larval intrusion in a group-living beetle *Trypoxylus dichotoma*

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Abstract Pupae of several insect species are known to generate air-borne sounds and/or substrate-borne vibrations, but the functions of the sounds/vibrations are not well understood. Here, we present the first evidence of vibratory communication between pupae and larvae of a group-living Japanese rhinoceros beetle *Trypoxylus dichotoma* which inhabits humus soil. The last-instar larvae of this beetle construct their own pupal cells to ensure normal pupation and eclosion. These cells are fragile and subject to damage from burrowing larvae because pupae and larvae co-inhabit the same patches of humus. In laboratory experiments, we demonstrated that pupal cells harboring live pupae were less likely to be broken by larvae than those harboring dead pupae. It was also demonstrated that pupae produced vibrations in response to larvae approaching the pupal cells. High-speed video and vibration analyses showed that pupae emitted 3–7 pulses at 1.3-s intervals by beating their pronotum against the inner wall of the pupal cell. The pupal vibration was of low frequency with a maximum energy at≈100 Hz. The drumming behavior was more frequently observed in the presence of an approaching larva than in its absence. When pupal vibrations were played back near to vacant artificial pupal cells, these cells were rarely disturbed by the larvae. These results provide evidence that pupae generate vibrations to deter conspecific larvae, thereby preventing damage to the cells. This larval response to pupal vibrations may have evolved through preexisting anti-predator and/or sib-killing-avoidance behavior.

Keywords Vibration · Rhinoceros beetle · Pupae · Altruism · Predator avoidance

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Introduction

Many insects use substrate-borne vibrations to communicate with conspecifics or heterospecifics (Cocroft and Rodríguez 2005; Cocroft and Hamel 2010). The vibratory signals have evolved in various contexts such as mating (Rodríguez et al. 2006; Sullivan-Beckers and Cocroft 2009), parental care (Cocroft 1999), cooperation (Cocroft 2005; Fletcher 2007), competition (Yack et al. 2001), and predator avoidance (Čokl and Virant-Doberlet 2003; Cocroft and Hamel 2010). While in most cases the signallers are individuals in the adult or larval stage (reviewed by Virant-Doberlet and Čokl 2004), pupae of some species are also known to produce vibrations or sounds (Hinton 1946, 1948; Travassos and Pierce 2000; Barbero et al. 2009). For example, Hinton reported sound production and mechanisms of sound production in pupae of Coleoptera and Lepidoptera and speculated defensive roles for the pupal acoustics (Hinton 1946, 1948). To date, however, no studies have experimentally demonstrated the functions of pupal signals except for the signals of the pupae of ant-tended lycaenid butterflies, whose sounds or

vibrations have been shown to induce attendance by ants (Travassos and Pierce 2000; Barbero et al. 2009).

In the present study, we dealt with the vibrations produced by the pupae of Japanese rhinoceros beetle Trypoxylus (Allomyrina) dichotoma (Coleoptera, Scarabaeidae, Dynastinae) in response to approaching larvae. Larvae of this species live in humus, ingesting it, or other wood-based detritus (Tsurumaki 1987). Female adults lay dozens of eggs in humus in July–August, and these eggs hatch within days (Tsurumaki 1987). By winter, the larvae develop to the last (the third) instar (20–30 g in weight; 6–8 cm in length; Tsurumaki 1987). They build their own pupal cells in May–July of the following year by compacting a mixture of fecal pellets and humus (Tsurumaki 1987). The pupal cells are oval (7–8 cm in major axis, 3–4 cm in minor axis) and built vertically underground at the same site where the larvae have grown (Tsurumaki 1987). The interiors of the pupal cells are essential for the extension and sclerotization of legs, wings, and horns (in the case of males), which accompany eclosion. However, the pupal cells are so fragile that they can be broken by disturbances such as intrusions by burrowing conspecific larvae. This species shows a highly aggregated distribution in the field (Kojima, personal observation). Larvae do not cannibalize pupae, but they actively move in the humus (Kojima, personal observation). Thus, frequent encounters of larvae with pupal cells are likely. The pupae and pre-pupae in pupal cells are vulnerable for an extended period, approximately 20 days (Tsurumaki 1987). The prolonged risk to pupae/pre-pupae may have prompted the evolution of mechanisms that prevent intrusions by conspecific larvae.

In this context, given that vibrations are produced by the pupae and pre-pupae of T. dichotoma in response to approaching larvae (Movie S1, S2), we hypothesized that the pupal vibrations function to deter burrowing conspecific larvae. This hypothesis predicts that (i) larvae and pupae should often be very close to one another in the field, (ii) larvae should avoid cells harboring live pupae, and (iii) pupal vibratory signals should be sufficient to deter burrowing larvae. We tested these predictions after analyzing the characteristics of the vibrations and the mechanism of their production in detail.

Materials and methods

Insects

The last-instar larvae of T. dichotoma used in experiments were collected from a number of humus patches in Tokyo, Kanagawa, and Ibaraki Prefectures, Japan, in spring 2009 and 2010. These larvae were reared at 25°C in a container filled with field-collected humus at a density of 1.5–2 individuals/ l humus. Larvae never cannibalized pupae or larvae.

Recordings and analyses of pupal vibrations

For recording from natural pupal cells, we introduced two or three larvae into a glass container ($35 \times 22 \times 25$ cm) filled with humus. After their pupation, we removed the humus over the pupal cells and created a small hole (<1-cm diameter) on the top of the cells. We stimulated the pupal abdomen by a gentle touch with a pair of tweezers. In response to the touch, most of the pupae rotated their abdomen three to seven times in succession. A continuation of successive rotating behaviors was defined as a bout. The substrate vibrations were recorded using a piezoelectric charge accelerometer (type 4381, Brüel & Kjær, Denmark) with a screw (5 mm in diameter $\times$ 25 mm) fixed on it, following the method of Mankin et al. (2000) with slight modifications. The screw was pushed into humus soil at a distance of 10.5 cm from the center of pupal cell. The signals from the accelerometer were amplified by a conditioning amplifier (type 2692 with a 1 Hz–10 kHz band-pass filter, Brüel & Kjær), digitized by an analog/ digital converter (PULSE, type 3560-B with a 0.7 Hz high-pass filter, Brüel & Kjær) at a sampling rate of 65.5 kHz (24 bits), and analyzed by the software PULSE (Brüel & Kjær). The accelerometer was calibrated for 10 m/s^2 using an accelerometer calibrator (type 4291, Brüel & Kjær). Two bouts were recorded from each pupa ($n=10$ bouts for five pupae: three females and two males) at 25–26°C.

In the following experiments, it was necessary to place pupal cells at defined positions in a container of humus soil. However, since natural pupal cells were fragile, it was difficult to move them from one place to another. To circumvent this problem, we formed "artificial" pupal cells in humus soil in a glass container ($35 \times 22 \times 25$ cm) by briefly thrusting a cylindrical tube (3 cm in diameter) into the soil to a depth of 5 cm. The artificial pupal cells (3 cm in diameter $\times$ 5 cm) looked very similar to natural pupal cells except that they were open at the top and more fragile. We introduced a pair of tweezers through the hole and stimulated the pupa mechanically. Two bouts of vibrations were recorded from each pupa ($n=16$ bouts for three female and five male pupae) at a distance of 10.5 cm from the center of pupal cell.

To record control stimuli for the playback experiment, we fixed pupal abdominal segments with superglue to prevent abdominal rotation. The immobilized pupae ($n=2$, one female and one male) were placed in artificial pupal cells and stimulated mechanically. Vibrations from the immobilized pupae were recorded as described above for the intact pupae. We examined if the characteristics of vibrations (acceleration and pulse interval) differ depending on the type of pupal cells (artificial or natural) or the sex of pupae. The effect of sex was investigated because pupae of this species show sexual dimorphism with the males having long horns. We randomly chose two pulses from each bout, which consisted

of 3–7 pulses, and averaged accelerations (m/s^2, zero-to-peak) of the pulses. To obtain average pulse interval, we measured intervals between the peaks of the two adjacent pulses. The means and standard deviations (SD) of the vibrational characteristics (acceleration of pulses and interval between pulses) were calculated from the average values for each individual. The effects of the sex of pupae and type of pupal cells (artificial or natural) on the vibrational characteristics were examined using a restricted maximum likelihood (REML)-based linear mixed effect model (LMM; Laird and Ware 1982). In this model, we entered pupal identification as a random effect term to take account of pseudo-replication. All statistical analyses in this study were performed using the software R (version 2.8.0; R Development Core Team 2010).

Power spectra of three consecutive pulses (2.4–3.1 s in total) were computed by PULSE software using a frequency resolution of 64 Hz and a Hanning window with maximum overlap. We obtained single spectra for the pupae examined and averaged them by sex or type of pupal cell. The averaged spectrum for background noise in four recordings (two females and two males) and the spectrum for two immobilized pupae were also obtained.

Simultaneous recordings of pupal rotating behavior and vibrations

We examined the mechanisms by which pupae produce the vibrations. Pupal rotating behavior and vibrations were recorded synchronously to examine the correspondence between pulse patterns and pupal rotating behavior. A single pupa ($n=2$, one female and one male) was introduced into a shot glass. The cavity of the shot glass was similar to the pupal cell in size and shape (4 cm in diameter, 5 cm in depth). We were able to observe and record the pupal behavior through the glass. The rotating behavior was recorded using a high-speed camera (FAST-CAM-512 PCI 32 K, Photron, Japan) with a Zoom-Nikkor lens (35–70 mm, f/3.3–4.5, Nikon, Japan) at 125 frames/s. Pupal vibrations were simultaneously recorded using a small accelerometer (type 4393 V, Brüel & Kjær) attached with beeswax to the shot glass, which served as an artificial pupal cell. The signals from the accelerometer were transmitted to the same system described in the previous section. The recorded videos were combined with the simultaneous vibrations and analyzed temporally using the software package TEMA 2D (Sweden).

Testing prediction (i): are pupal cells close to larvae in natural habitats?

To examine the closeness between larvae and pupal cells in natural habitats, we investigated the distribution of pupal cells and larvae in a patch of humus (≈160 cm in length, ≈90 cm in width), located in a broadleaf deciduous forest, in Kawasaki City, Japan (35°60 N and 139°50 E). We knew beforehand that many larvae inhabit this site. In May 2009, we placed five quadrats (25×25 cm) in the humus patch randomly with a >5 cm space between them. We shoveled the humus to a depth of 25 cm and recorded the three-dimensional coordinates of the head of larvae and the top of pupal cells in each quadrat. We also counted the number of other grossly visible animals in each quadrat.

Testing prediction (ii): do larvae avoid live pupae?

To examine the interaction between pupae and larvae, we conducted four types of experiments. All laboratory experiments other than recordings of vibrations were done in the dark at 25–26°C.

Experiment with natural pupal cells

We examined if a larva broke a natural pupal cell that harbored a live pupa. Single last-instar larvae were transferred to transparent plastic vials (8 cm in diameter× 12 cm) filled with humus. They built a pupal cell adjoining the lateral side of the vial. Thus, we were able to see inside of the pupal cell through the wall. After 3–6 days of pupation, we traced the outline of the pupal cell on the vial using a red marker, which served as a marking of the cells. After the introduction of a single last-instar larva, the vials were maintained in the dark at 25°C for 6 h, and then the pupal cells were visually inspected. We rechecked the results of the inspection when we removed the larva from the vial after the experiment. For controls, we used dead pupae. After 3–6 days of pupation, we froze the vials at −20°C for 3 h to sacrifice the pupae and then maintained them at room temperature for about 4 h. The control vials were subjected to experiments as described above. Chi-square test of independence was used to compare the percentages of larvae that broke cells in the control and test groups. The sample sizes for the non-treated and frozen vials were 11 and 9, respectively.

Experiment with artificial pupal cells

We examined if a larva broke an artificial pupal cell containing a pupa. A rectangular plastic container (30×7× 7 cm) was filled with humus. We prepared two artificial pupal cells, one each 10.5 cm left and right of center. In this experimental system, larvae were not able to move to the end of the container without breaking an artificial cell. We examined whether larvae broke artificial pupal cells containing a live pupa. We introduced a single pupa into each of the two artificial cells prepared in the container as described above. Then we placed a larva at the center of the

container and let it burrow into the soil. After 60 min, we examined if the artificial pupal cells were broken by the burrowing larva. The pupal pairs used in each trial were chosen randomly from 26 individuals. For controls, we introduced a dummy pupa (an oval stone, similar to a pupa in size and weight, covered with a sheet of Kimwipes®) to both artificial cells. Chi-square test of independence was used to compare the percentages of larvae that broke cells in the control and test groups. The sample size was 29 for the treatment group and 65 for the control group.

Pupal response to larvae

Using the same experimental system as above, we recorded the rotating behavior of the pupae in artificial pupal cells using a digital video camera (Handycam DCR-DVD403, Sony, Japan). We compared the number of bouts (a series of rotations) performed by each pupa in 60 min in the presence ($n=10$ trials using 20 pupae) or absence ($n=9$ trials using 18 pupae) of a burrowing larva. Zero-inflated Poisson regression (Lambert 1992) was used to analyze the effect of the presence of a larva on pupal behavior.

How far do the larvae move away from pupae?

We also conducted choice experiments to examine how far the larvae moved away from pupae using the same experimental system as above. If larvae actively move away from vibrating pupae, most larvae will be found far from the cell with a pupa. We introduced a pupa into one of the artificial pupal cells and a dummy pupa into the other. Then we placed a larva at the center of the container, and let it burrow into the soil. After 60 min, we recorded the position of the larval head ($n=44$ trials). In control experiments, a single dummy pupa was placed in each of the two artificial cells ($n=44$ trials). The effect of the presence of a pupa on the damage of pupal cell was tested by Chi-square test. The larval distributions in the control and test groups were compared using two-sample Kolmogorov–Smirnov (KS) test.

Testing prediction (iii): do pupal vibrations deter larvae? (Playback experiment)

To examine whether larvae respond to pupal vibrations, vibrations were played back near to vacant artificial pupal cells using the experimental system shown in Fig. 1. Two artificial pupal cells were prepared in a rectangular container as described above. The tips of a U-shaped gadget made of a stainless-steel strip ($310 \times 10 \times 2$ mm) were placed under the artificial cells (15 mm from the bottom) through the holes made at the bottom of the container. The container (750 g including the gadget and soil) was suspended with a plastic string from the ceiling of a one-side-opened soundproof box
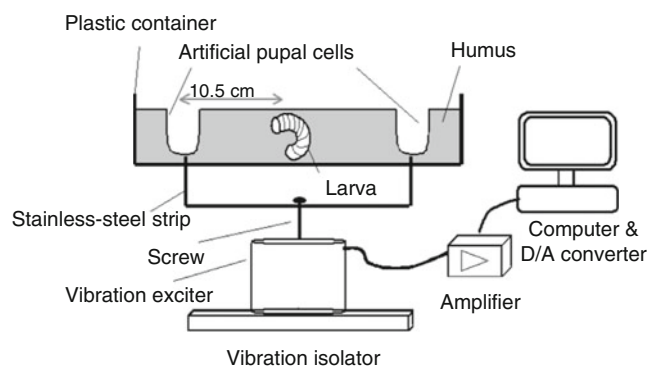
Fig. 1 Experimental system for checking the effect of played back vibrations on the behavior of larvae. For each trial, a larva was placed at the center of the container, and the vibrations were played continuously at intervals of 10 s for 60 min. At the end of trials, it was checked whether a larva in the humus had broken artificial pupal cells

($90 \times 90 \times 70$ cm). The gadget was fixed with a screw (5 mm in diameter $\times 25$ mm) to a vibration exciter (type 4809, Brüel & Kjær) placed on a desktop vibration isolator (UM-0405, Nippon Boushin, Japan). The vibrations were generated by the exciter connected to a power amplifier (type 2718, Brüel & Kjær). For playback experiments, we clipped one bout (ca. 4.5–7 sec) containing 3–7 pulses from a recording file (see "Recordings and analyses of pupal vibrations"), and converted it into a WAV file using the PULSE software. The signals of the edited file were transmitted to the exciter via a DAQ card 6062E (12 bits, National Instruments) at a 44.1-kHz sampling rate by RECORDER software (Avisoft, Germany). The output from the exciter was adjusted so that the acceleration of vibrations at the center of the container was equal to that of the original recordings. In addition to the acceleration, we confirmed that frequency and temporal characteristics were not different from those of the original recordings. The frequency response of the container vibrated by the exciter was relatively flat over the frequency range of interest (10–5000 Hz).

The playback experiment was done in the dark at 25–26° C. To start a trial, a larva was placed at the center of the container to let it burrow into the humus. The vibrations were played back for 60 min at intervals of 10 s, which is close to the mean interval between the bouts of vibrations (see "Characteristics of pupal vibrations" in "Results"). At the end of each trial, it was checked whether the artificial pupal cells were broken by the burrowing larva. For playbacks, three files from recordings of pupae in natural pupal cells and four from recordings of pupae in artificial pupal cells were chosen. Trials were done for 6–8 larvae per file, and the total sample size was 51 larvae. For controls, the vibrations from immobilized pupae or the background noise of recordings of pupal vibrations were used. We edited 5-s files by clipping recordings and played them as described above. Trials were done for 8 and 10 larvae per file for the

immobilized pupae and background noise, respectively. The total sample size was 18 and 30 larvae, respectively. We used Chi-square test with Bonferroni correction in the analyses of playback experiments.

Results

Characteristics of pupal vibrations

Pupal vibrations produced in response to contact stimuli were characterized. The vibrations consisted of bouts that comprised several pulses (Fig. 2a). In 63% of the trials (24 measurements with 6 pupae), the bouts were repeated two to four times at intervals of 9.0 s (SD=3.4 s, n=6 pupae), while a single bout was generated in the rest of the trials. Synchronous recordings of the vibration and movement of pupae with high-speed video suggested that these pulses were emitted when pupae beat the dorsal side of their prothorax (i.e., pronotum) against the inner wall of pupal cells (Movie S1). The interval between two successive pulses was 1.3 s (SD=0.18, 52 measurements with 13 pupae; Fig. 2a), which was equivalent to the time required for pupae to complete a rotation inside their pupal cells (Movie S1). Pre-pupae were also observed to rotate and

beat the inner wall of pupal cells with the pronotum (Movie S2). The pulse interval for pre-pupae was similar to that for pupae (ca. 1.6 s, Movie S2). The sex of pupae and type of pupal cells (artificial or natural) did not affect the interval between pulses for pupal vibrations (LMM; sex: t=−0.16, p=0.87; type of pupal cell: t=−0.42, p=0.68). The average acceleration of pulses in pupal vibrations was 0.26 m/s^2 (SD=0.12, 52 measurements with 13 pupae; Fig. 2a) at a distance of 10.5 cm from the cell. The sex of pupae and type of pupal cells did not affect the acceleration (LMM; sex: t=−0.64, p=0.53; type of pupal cells: t=−0.065, p=0.95).

Recorded pupal vibrations were in the low-frequency range, mostly below 500 Hz (Fig. 2c). The frequency with maximum energy was ca. 100 Hz. The spectra of four groups differing in sex and the type of cells were similar (Fig. S1). The spectrum of vibrations from immobilized pupae was similar to that of background noise (Fig. S1).

Testing prediction (i): are pupal cells close to larvae in natural habitats?

The feral distribution of pupal cells and larvae in soil was surveyed by the quadrat method. Each quadrat (25×25 cm) contained, on average, 4.2 larvae and 12.6 pupal cells. Every pupal cell contained a pre-pupa or a pupa. Pupal cells

Fig. 2 Vibrations generated by pupae. **a** An oscillogram of vibration produced by a male pupa in a natural pupal cell. The bout contained six pulses indicated by arrowheads. **b** An oscillogram of a magnified pulse between *broken lines* in **a**. **c** Averaged power spectra of three consecutive pulses in a bout from natural pupal cells harboring a male pupa (*bold black line*), and averaged power spectra of background noise from individual recordings (*thin grey line*). Sample size is shown in *parentheses*
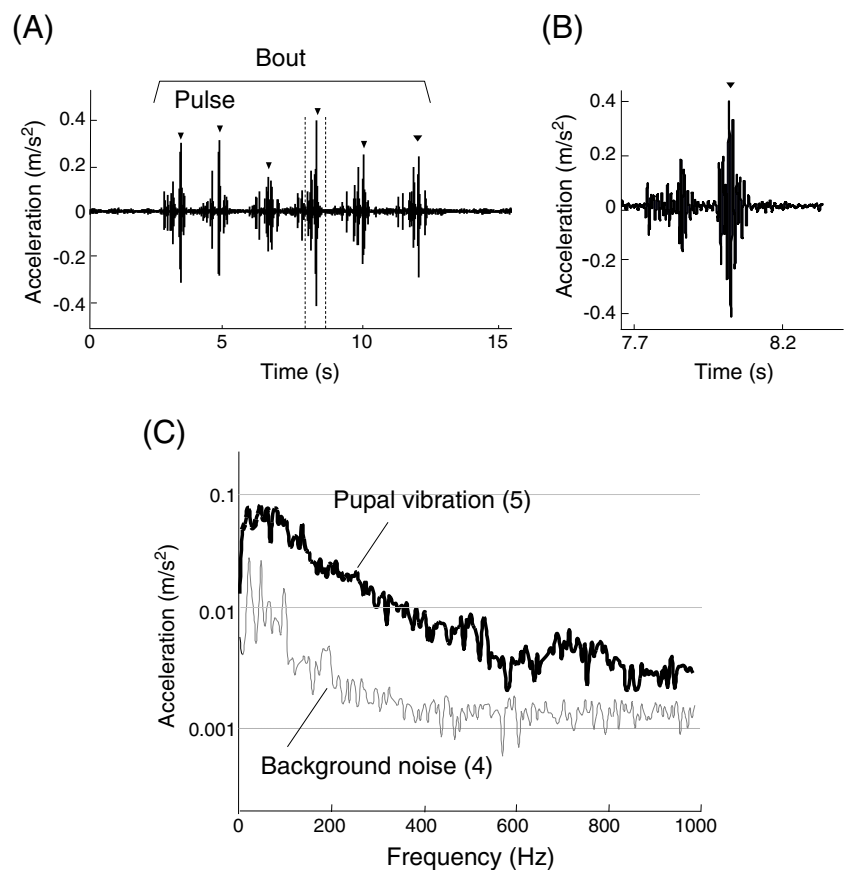
were distributed in close proximity to larvae (Fig. 3a, b): the average distance between each larva and its nearest pupal cell was 6.4 cm (SD=3.3 cm, n=21), and the shortest distance was 2.8 cm.

Other invertebrate species, earthworms (*Eisenia* sp.), the woodlouse *Porcellio scaber*, the centipede *Bothropolys asperatus*, and larvae of flower beetles (Cetoniinae), were also found, but at low density (0.4, 0.8, 0.2, 0.2 individuals/ quadrat/respective species).

Testing prediction (ii): do larvae avoid live pupae?

Experiment with natural pupal cells

A pupal cell harboring a live pupa was seldom damaged by a larva in the same vial (proportion of cells destroyed by larvae, 9%, n=11). Pupal cells harboring dead pupae were significantly more vulnerable to larvae than were the controls (89%, n=9, χ^2=13, p<0.001). In one case, a male pupa in a broken pupal cell was found to be injured and bleeding from the horn (Fig. S2A).

Experiment with artificial pupal cells

In no-choice experiments, an artificial pupal cell with a live pupa was not broken by a larva in the same plastic
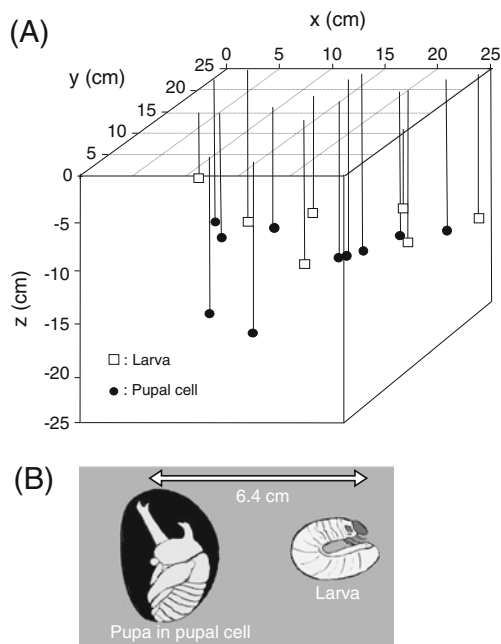
Fig. 3 Distribution of pupal cells and larvae in a patch of humus located in a broadleaf deciduous forest. **a** Typical example of the distribution of pupal cells and larvae in a quadrat. A similar pattern of distribution was observed in the other four quadrats. **b** A pupa in a pupal cell and a larva. The average distance between pupal cells and larvae in their natural habitat was 6.4 cm

container in any replicates (0%, n=29; Fig. 4a). An artificial cell with a dummy pupa had a significantly higher probability of being damaged than the controls (52%, n=65, χ^2=24, p<0.001; Fig. 4a).
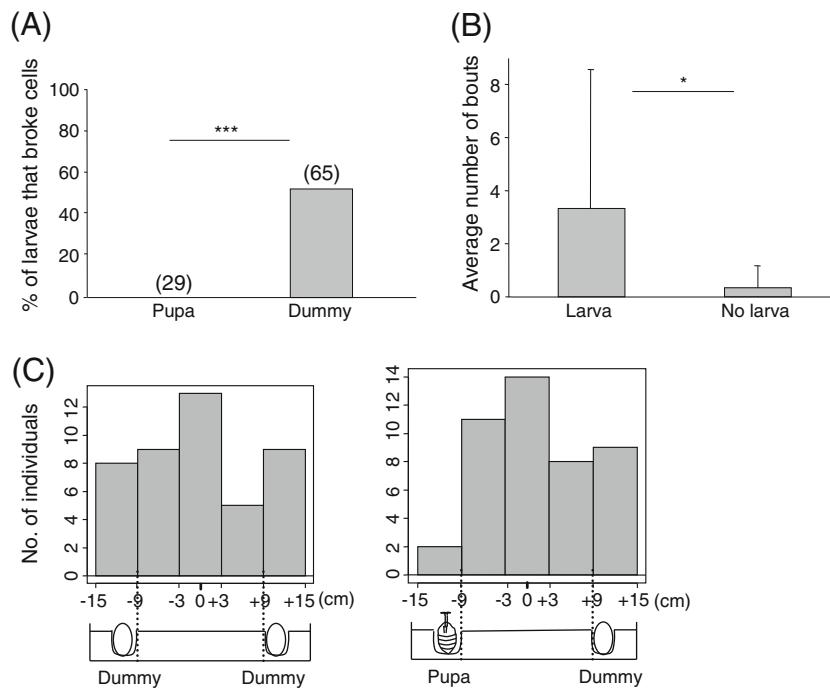
Pupal response to larvae

Pupae were observed to generate vibrations more frequently in the presence of a larva (3.4 times/h, n=20, no cells broken) than in its absence (0.3 times/h, n=18, zero-inflated Poisson regression model; z=2.3, p=0.02; Fig. 4b).

How far do the larvae move away from pupae?

In choice experiments, the artificial pupal cells with a dummy were more often broken (23%, n=44) by larvae than those with a live pupa (5%, n=44; χ^2=6.2, p=0.01). Figure 4c shows histograms of larval distribution in the control experiments (left) and choice experiments (right). Since the presence of a pupa appeared to have affected the distribution of larvae only in a short range (±3 cm), the data for the leftmost bins in the histograms were compared separately from the others. Significantly smaller number of larvae were present in the leftmost bin in the choice experiments (Fig. 4c, right graph) than in the control experiments (Fig. 4c, left graph; Chi-square test, n=44, χ^2=4.1, p=0.04). When the leftmost bins were excluded, the pattern of distribution in the choice experiments was similar to that in the controls (two-sample KS test, n=78, χ^2=0.1, p=1.0), suggesting that the pupae did not repel the larvae but deterred their approaching.

Testing prediction (iii): do pupal vibrations deter larvae? (Playback experiment)

In playback experiments, a significant difference in the percentage of trials in which cells were broken by larvae was found among the three groups (vibrations from intact pupae, background noise, and immobilized pupae; χ^2=34, p<0.001). In only 3 of 51 trials (6%) were artificial pupal cells with pupal vibrations damaged (Fig. 5): one of 24 larvae broke an artificial cell with the playing back of pupal vibrations recorded from artificial cells with a pupa, and 2 of 27 larvae did so with the playing of vibrations recorded from natural pupal cells. On the other hand, artificial cells with background noise or vibrations recorded from artificial cells with an immobilized pupa were more frequently damaged than those with recordings of intact pupae (background noise, 60%, n=30, χ^2=28, p<0.0003; immobilized pupae, 62%, n=18, χ^2=25, p<0.0003). No significant difference was detected between the groups with background noise and vibrations from immobilized pupae (χ^2=0.03, p=0.87).

Fig. 4 Larval response to pupae in artificial pupal cells. **a** Percentage of larvae that broke an artificial pupal cell when a pupa or dummy was in the cell inside a plastic container. Sample size is shown in *parentheses*. ***$p < 0.001$ by χ^2 test. **b** Average number of bouts for pupal rotating behavior per hour in the presence ($n = 20$ trials) or absence ($n = 18$ trials) of a larva. *$p = 0.02$ by zero-inflated Poisson regression. Error bar: standard 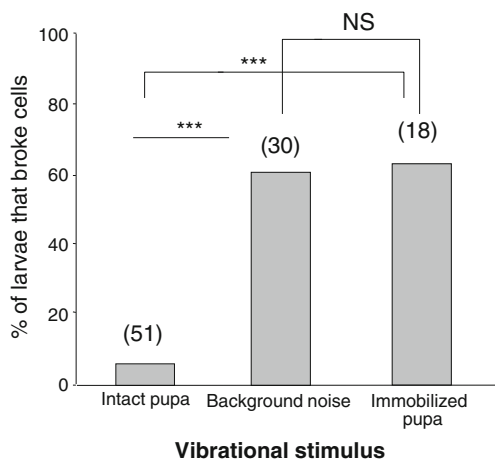deviation. **c** Histograms of larval positions when a dummy was placed in both artificial pupal cells (*left*), and when a pupa was placed in one cell and a dummy in the other (*right*). The distance at the end of trials from the initial position (0 cm) for 44 larvae is shown. In the *right* histogram, positive values show that larvae moved away from a pupa, and negative values show that larvae approached a pupa

Discussion

It was shown that pupae of *T. dichotoma* in pupal cells rotated their abdomen to generate substrate vibrations in

Fig. 5 Percentage of larvae that broke an artificial pupal cell with reproductions of vibrational recordings. Three types of vibrational playbacks were presented: vibrations from intact pupa, background noise from intact pupa, and vibrations of immobilized pupae. Sample size is shown in *parentheses*. *NS* Not significant. *** Significant ($\alpha = 0.001$) by χ^2 test with Bonferroni correction

response to approaching larvae. We also demonstrated that larvae avoided pupal cells in their way when pupae in the cells generated vibrations. Thus, pupal vibrations of this species probably function as deterring signals to the larvae. These results provide, to our knowledge, the first experimental evidence for conspecific communication mediated by pupal vibrations.

Larvae of this species exhibit a highly aggregated distribution, possibly because of their restricted source of food and the adults' oviposition habits. As shown by our field data (Fig. 3a, b), larvae in the same patch do not pupate synchronously. Consequently, pupal cells are in close proximity to many larvae. In the field, the average distance between pupal cells and larvae (6.4 cm) was shorter than the body length of the larvae (7.7 cm; Tsurumaki 1987). Larvae do not cannibalize the pupae, but given the high activity of larvae and fragility of pupal cells, pupal cells are at high risk of being accidentally damaged by neighboring larvae. The pupa or pre-pupa in such cells was found to be injured, resulting in death (Fig. S2A) or serious deformity due to a failure of pupation and eclosion (Fig. S2B). Thus, we suggest that, under natural conditions, deterring signals of pupae emitted against conspecific larvae are important in successful development toward adulthood.

Conspecific vibratory signals for cooperative or competitive interactions are common in group-living insects (reviewed by Cocroft and Hamel 2010). The vibratory communication can occur between individuals in the same developmental stage or in different stages; for example, it occurs between larvae in a sawfly (Fletcher 2007) and between adult females and their offspring in a subsocial treehopper (Cocroft 1999). To date, however, intraspecific vibratory communication between larvae and pupae has not been reported. Pupal vibrations in this species have low-frequency components: the peak frequency was ca. 100 Hz with an acceleration of ca. 0.26 m/s^2 (Fig. 1a, b). Some insects are known to have sense organs for vibrations (e.g., chordotonal organs and mechanosensilla), which well respond to low-frequency vibrations including the 100-Hz level (McIver 1985; Field and Matheson 1998). Playback experiments showed that vacant artificial pupal cells with pupal vibrations were rarely disturbed, indicating that vibration is sufficient for the protection of pupal cells from larvae. Given that natural pupal cells harboring dead pupae were damaged by larvae at a higher rate than those containing live pupae, it is obvious that the pupal cells are not structurally strong enough to resist disturbance by larvae.

We found that pupae rotated their abdomen by intersegmental extension and beat the pronotum against the wall of the pupal cell, thereby generating vibratory signals (Movie S1). Pupal abdominal rotation and/or intersegmental extension is widespread among endopterygotes (Hinton 1946, 1948), although the patterns of behavior vary widely from species to species (Hinton 1948). The rotating behavior and intersegmental pinching devices so called gin traps in other beetle species have been suggested to have anti-predator functions (Hinton 1946; Eisner and Eisner 1992; Ichikawa and Kurauchi 2009). In *T. dichotoma*, the pupal vibrations or the abdominal rotation behavior may function not only as signals to conspecific larvae but also as a defense against predators and other intruders.

Although larvae of this species avoided pupal cells when they received vibratory signals, they did not rapidly move away from the source of the vibration (the pupal cell); they were sometimes found close to the pupal cell both in the laboratory choice experiments (Fig. 4c) and under natural conditions (Fig. 3a). Pupal vibrations may arrest the movement of larvae rather than repel them from the pupal cells.

Most insects have sensory organs to detect the vibratory noise generated by the movements of predators, prey, or conspecific mates (Stumpner and von Helversen 2001; Hill 2009). Upon the detection of vibratory cues, prey species show evasive behavior such as escaping, freezing, or a stereotyped startle response (Stumpner and von Helversen 2001; Hill 2009). Pupae of *T. dichotoma* may have

exploited the anti-predatory response for the protection of their cells (cf. sensory traps; Edwards and Yu 2007). The response of larvae to pupal vibrations seems to confer little benefit to the larvae themselves. However, if discrimination of pupal signals from predatory vibrations imposes substantial costs on larvae due to sensory constraints, the response may be evolutionarily maintained along with pupal signals. Moreover, the communication system may have been maintained partly via kin selection. Adult females lay dozens of eggs at one site (Tsurumaki 1987). Moreover, hatched larvae probably remain at the same location until they become adults because the humus patches they inhabit are usually isolated from each other. Therefore, siblings are likely to live close together. In such a situation, kin selection could favor the current larval response because it reduces the fitness costs associated with "killing" sib pupae (cf. altruism based on limited dispersal; Hamilton 1964a, b; Gardner et al. 2010). Further studies are required to elucidate the evolution of "underground" vibratory communication in this species.

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