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Research

Twittering Pupae of Papilionid and Nymphalid Butterflies (Lepidoptera): Novel Structures and Sounds

Patrick Dolle,¹ Philipp Klein,¹ Ottmar W. Fischer,¹ Hans-Ulrich Schnitzler,² Lawrence E. Gilbert,³ and Michael Boppré^{1,4}

¹Forstzoologie und Entomologie, Albert-Ludwigs-Universität, D-79085 Freiburg i.Br., Germany, ²Institut für Neurobiologie, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany, ³Department of Integrative Biology, The University of Texas at Austin, 2415 Speedway, Austin, TX 78712, and ⁴Corresponding author, e-mail: boppre@fzi.uni-freiburg.de

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Abstract

Pupae of numerous Papilionidae and Nymphalidae produce twitter sounds when wriggling in response to mechanical stimulation. The structural basis comprises distinct pairs of sound-producing organs (SPOs) located at intersegmental membranes of the abdomen. They differ—as the twitters do—in sampled taxa of Papilioninae, Epicaliini, and Heliconiini. The opposing sculptured cuticular sound plates (SPs) of each SPO appear structurally the same but are actually mirror-images of each other. Results suggest that sounds are not generated by stridulation (friction of a file and a scraper) but when these inversely sculptured and interlocking surfaces separate during pupal wriggling, representing a stick-slip mechanism. Twitter sounds comprise series of short broadband pulses with the main energy in the frequency range 3–13 kHz; they can be heard by humans but extend into ultrasonic frequencies up to 100 kHz.

Key words: sound plate, stridulation, stick-slip friction, antipredator device, protest sound

The first report on butterfly pupae producing sounds seems to be that of Sealy (1875): ‘... The pupa [of *Troides minos* (Cramer), as *Ornithoptera amphrisius*] possesses the power of making a curious noise, like “pha, pha,” and makes it very loudly when touched; ...; Davidson and Aitken (1890) called it ‘a husky squeaking noise’, produced apparently by friction of the abdominal rings—but the subject seems not to have received further attention.

Acoustic communication in insects remains a subject of much interest (Alexander 1957, 1967; Frings and Frings 1958; Masters 1979, 1980; Ewing 1989; Drosopoulos and Claridge 2006; Hedwig 2014) but butterflies have been mostly ignored. Reports on sound production in adult Lepidoptera mainly relate to moths and are discussed in the context of territoriality (Lees 1992), antipredator and courtship signals (for reviews, see Conner 1999; Conner and Corcoran 2012; Greenfield 2014; Nakano et al. 2015; ter Hofstede and Ratcliffe 2016), and much less to butterflies (e.g., *Hamadryas* [Yack et al. 2000; Marini-Filho and Benson 2010]; *Heliconius* [Hay-Roe and Mankin 2004]). With respect to butterfly immatures, larval and pupal acoustic signaling is known and has been studied in the closely related Lycaenidae and Riodinidae (Downey 1966, 1967; Hoegh-Guldberg 1972; Downey and Allyn 1973, 1978; DeVries 1991; Pierce et al. 2002; Álvarez et al. 2014, 2018; Ballmer and Wright 2014; Riva et al. 2017; Schönrogge et al. 2017). Hinton (1948) described different ways of sound production in pupae and

mentioned ‘hissing sounds’ in a variety of Lepidoptera, including the papilionid *Papilio polytes* Linnaeus (Hinton 1955); Hinton (1969) also illustrated a ‘stridulatory file’ in pupae of *Troides aceracus* [sic, recte *aeacus*] (C. & R. Felder) (Papilionidae: Papilioninae); Alexander (1961), Gilbert (1991 personal communication to M.B.) and Hay-Roe (2006) mentioned audible sounds from pupae of some *Heliconius* spp. (Nymphalidae: Heliconiinae). Penz (1999) and Penz and Pegg (2003) used presence or absence of ‘a stridulatory organ’ as a character in phylogenetic analyses of Heliconiinae. Surprisingly, for Heliconiinae no details have been provided to date with respect to sounds and/or related structures; reviews on Heliconiinae (Beebe et al. 1960; Brown 1981; Brower 1997) and a series of papers giving very detailed documentation of morphological characters of early instars of several heliconiine genera (Vargas et al. 2014 and refs therein) do not even mention pupal sound organs.

The Butterfly House Industry ships millions of butterfly pupae from producers to exhibitors (Boppré and Vane-Wright 2012). When pupae are handled during packing or unpacking, many wriggle, and sometimes ‘protest sounds’ (sensu Alexander 1957) can be heard by humans. Here, we report on sounds in pupae of 35 species in 13 genera of Papilionidae and Nymphalidae, erratically selected from the market of the Butterfly House Industry, and principally document and compare the sounds as well as the underlying structures (sound-producing organs [SPOs]) for representative species.

Material and Methods

Initial studies were conducted at the butterfly farm *El Bosque Nuevo* (EBN 2018) near Santa Cecilia, Guanacaste, Costa Rica. Later, several species were purchased from pupae traders. Species studied are listed in Fig. 1.

For obtaining an overview, each available species was checked for sound emission: pupae were mechanically stimulated by hand (held at their thoraces) or stroked with a paintbrush. Any sounds emitted were made audible with a Baton bat detector (Batbox Ltd., Steyning, UK) which covers a frequency range of 20–120 kHz using a frequency division method (in this case a signal is divided by 10) to make ultrasounds audible for humans. Pupae reluctant to move could be forcibly flexed and, in case, sounds elicited in this way. Pupae and, in particular, exuviae were searched for presumed SPOs under a stereo microscope.

Following this screening, the Baton bat detector was attached to a recorder (Mc Crypt DR-3; Conrad Electronic SE, Hirschau, Germany); recordings were made with a sample rate of 44.1 kHz and stored as 16 bit.wav files on a SD card. They were displayed with Audacity 2.0.6 (Audacity® 2014) on a Macbook Pro. Of species representing different types of SPOs, the structures were morphologically characterized in detail and sounds recorded and analyzed using more sophisticated equipment (below).

Morphology

For morphological studies, pupae were flexed to expose the presumed SPOs; exuviae of freshly hatched specimens were fixed with pins and dried for later examination; already dry exuviae were softened in a humid chamber, prepared accordingly, and dried again.

Macrographs of pupae and exuvia preparations were taken with a Keyence VHX-700FD digital microscope (Keyence Microscope Europe) equipped with a VH-Z20R/VH-Z20W zoom lens 20–200× and a polarization filter OP-87429 or with a zoom lens 100–1,000×.

For scanning electron microscopy (SEM) of SPOs, the respective regions were cut from exuviae, glued on aluminium pin stub specimen mounts (Plano GmbH, Wetzlar, Germany) using an adhesive disc (Leit-Tab; Plano GmbH, Wetzlar, Germany), gold-coated in an Edwards sputter coater (Edwards, Kirchheim, Germany), and studied with a Zeiss DSM 940A (Zeiss, Oberkochen, Germany) equipped with a DISS5 unit (point electronic, Halle, Germany).

Digital images were, when necessary, brightness adjusted using Adobe Photoshop CS6 and arranged with Adobe InDesign CS6 on a MacBook Pro.

Sound Recording and Analyses

Signals were recorded with a custom-made ultrasound recording system (PC-Tape, Department of Animal Physiology, University of Tübingen, Germany) and a Knowles Electronics microphone FG-23329 (Itasca, IL, USA). This microphone is most sensitive for signals with frequencies up to 10 kHz, but also covers the frequency range up to 110 kHz where its sensitivity is reduced by 30 dB. The sounds were digitized with a sampling rate of 256 kHz and stored with a post trigger system as 16-bit.wav files with a duration of 5 s on a Sony VAIO laptop. Pupae were held manually by the thorax. The tip of the microphone was positioned ventrally between segments 4/5 and 5/6 (Papilioninae), 4/5 (Epicaliini), or 6/7 (Heliconiini) at a distance of 2 cm. To check if sounds originated from the presumed SPOs, selected species were treated with immersion oil (L4085, agar scientific, GB-Stanstead) to mute these organs. With the exception of single samples for *Battus polydamas* (Linnaeus) and *Dryas iulia* (Fabricius), recordings of two to three individuals per species were selected for the description.

Signals were displayed and analyzed with custom-written bioacoustic software (Selena; Department of Animal Physiology, University of Tübingen, Germany) as direct measurements from oscillograms and color spectrograms with a dynamic range of 70 dB and a maximum

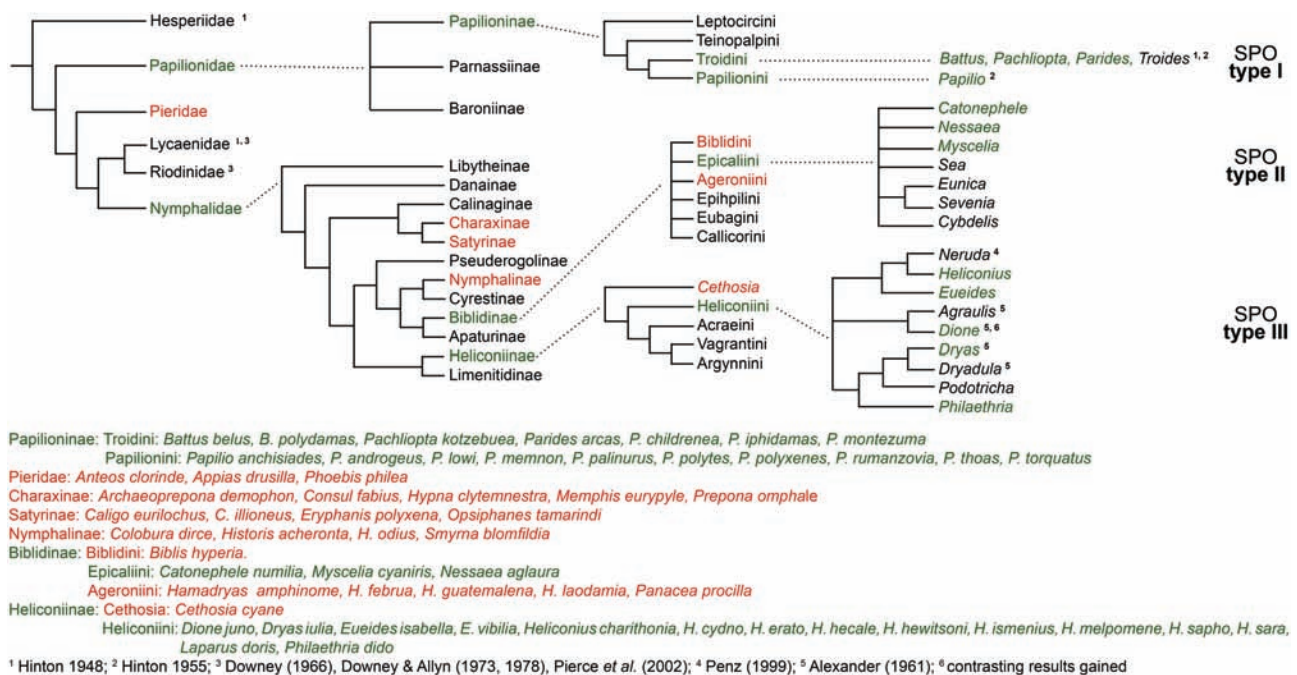


Fig. 1. Species studied for pupal SPOs and sounds, projected into the current phylogeny of butterflies (Papilionoidea) of the Tree of Life Web Project (2018). green: taxa with SPOs; red: taxa with movable abdominal segments but no sound or SPOs found; black: taxa not studied here; footnotes provide references to papers mentioning pupal sound in respective taxa. Note that all higher taxa, including those marked red or green, are insufficiently studied—it is shown that SPOs are widely distributed in Papilionoidea but a phylogenetic discussion is not yet possible.

frequency of 128 kHz. The spectrograms were calculated using a Fast Fourier Transformation of 128 frames and the weighting function Hann.

Results

General Findings

In 35 out of 58 available species of Papilionidae and Nymphalidae, we could amplify and hear sounds with the Baton bat detector when a pupa was wriggling, i.e., it moved its abdominal segments so that the intersegmental membranes become exposed (Figs 2 and 3). Twittering can easily be heard by younger persons, when holding a wriggling pupa close (1–2 cm) to an ear. In the other 23 species, including some Pieridae and several subfamilies of the Nymphalidae, either no sound could be recorded, no movement of the abdomen could be stimulated, or no SPOs were found (see Fig. 1).

Microscopical inspection revealed apparent SPOs on distinct areas of the intersegmental membranes. In resting pupae, these are always undetectable; only when the abdominal segments are flexed they do show up (Figs 2 and 3). The size of the SPOs observed ranged from 0.4 to 7.4 mm², depending on the species. Thus, in most cases, these structures are only detectable with optical aid. Due to the different types of attachment pupae move differently, and this relates to the position of the SPOs: girdled Papilionidae make dorso-ventral movements and activate their ventro-lateral SPs when ventral intersegmental membranes become simultaneously exposed (Figs 2A and 3A and C); suspended Nymphalidae swing laterally and thus activate lateral SPOs; in these pupae, the intersegmental membranes open alternately (Figs 2B and 3E,H,K,M).

Morphology

In general, pupal SPOs—four ventro-lateral ones in Papilionidae and two lateral ones in Nymphalidae—represent cuticular modifications of the intersegmental membranes which we call sound plates (SP); an anterior SP (aSP, closer to thorax) opposes a posterior plate (pSP, closer to abdominal tip) (Fig. 3).

Taking into account the position and fine structure of SPOs, in our samples, three types (I–III) of SPOs could be differentiated and characterized (Fig. 3); they demonstrate general differences between Papilionidae and Nymphalidae but always exhibit similar—although inverse—microstructures of aSPs and pSPs.

Type I SPOs in Papilionidae: Papilioninae: Troidini (*Pachliopta kotzebuea* (Eschscholtz), *Parides montezuma* (Westwood)), *Papilionini* (*Papilio polytes*) show up ventro-laterally between abdominal segments 4/5 and 5/6 (Figs 3A–E and 4). These four organs are made of transverse ± regular ridges on aSPs (Fig. 4C) and pSPs (Fig. 4D); the SPOs between abdominal segments 4/5 are larger and more prominent than those between segments 5/6 (Fig. 3A and C). There are great differences in the size of the organs between the species (1.5–7.4 mm²) and also in the number and dimensions of the ridges (Fig. 4G–J).

Type II SPOs in Nymphalidae: Biblidinae: Epicaliini (*Catonephele numilia* (Cramer), *Nessaea aglaura* (Doubleday), *Myscelia cyaniris* Doubleday) show up laterally between segments 4/5 in line with spiracles (Figs 3F–J and 5). The size of the two organs varies in the three observed species (2.1–3.0 mm²) and they consist of very regularly arranged oval elevations on pSPs (Fig. 5C) and depressions on aSPs (Fig. 5D).

Type III SPOs in Nymphalidae: Heliconiinae: Heliconiini (*Heliconius hecale* (Fabricius), *Philaethria dido* (Linnaeus), *Laparus doris* (Linnaeus), *Dryas iulia*) show up laterally between segments 6/7 in line with spiracles (Figs 3K–O and 6), and are the smallest (0.4–1.3 mm²) of all SPOs observed. As in type II SPOs, both organs consist of very regularly arranged ± boat-shaped elevations on pSPs (Fig. 6C) and depressions on aSPs (Fig. 6D); in Epicaliini, the depressions and elevations are more round, scale-like and, in particular, located between different abdominal segments (Fig. 3F and K). Within the tribe, the structures of the depressions and elevations vary with respect to size and shape; however they share the same basic structures (Fig. 6K–P).

Fracturing the organs transversely reveals that the sculptures making up the SPOs are solid multilayered chitin (Figs 4E and F, 5E and F, 6E and F). The two parts of the intersegmental membranes in question differ in thickness: the pSP on a more flexible membrane pulls away from the aSP on a more stiffly sclerotized membrane (see Fig. 10).

The broad characterizations of SPOs given above hold true for all species and many dozens of individuals checked for presence of SPOs (Fig. 1), although, species can be distinguished by certain structural details. However, the viewing angle significantly affects the appearance of the microstructures (see Fig. 6G–J). This and the need for artificial opening of the tiny and fragile SPOs as well as

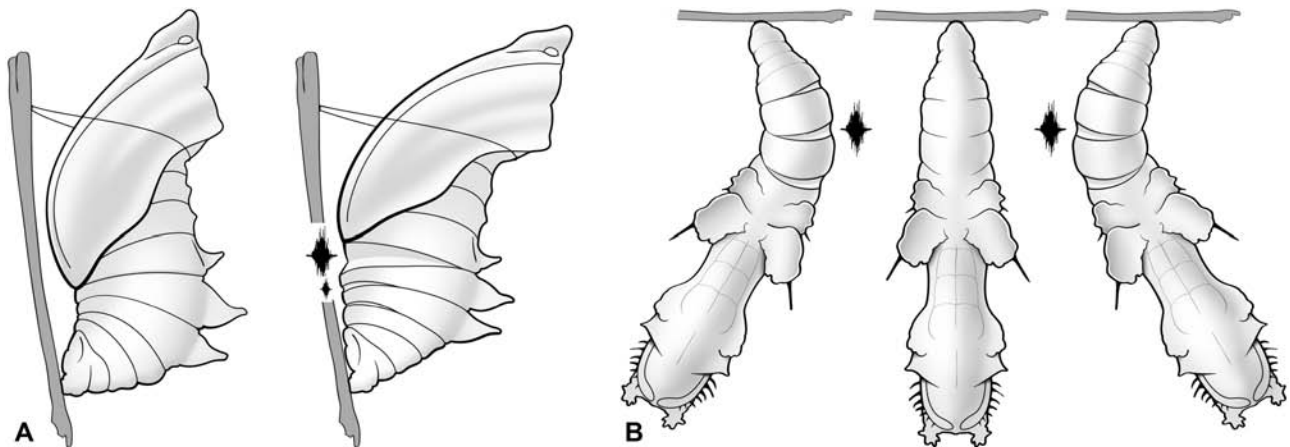


Fig. 2. Sketches of wriggling in pupae of a girdled papilionid (A, viewed laterally) and a suspended nymphalid (B, viewed dorsally), indicating their freedom to move dorso-ventrally in Papilionidae and laterally in Nymphalidae as well as the opening of intersegmental membranes where the SPOs are located (twitter symbols). Note that in Papilioninae which wriggle dorso-ventrally there are four ventro-lateral SPOs acting synchronously while in Nymphalidae there are two lateral SPOs acting alternately (see Fig. 3).

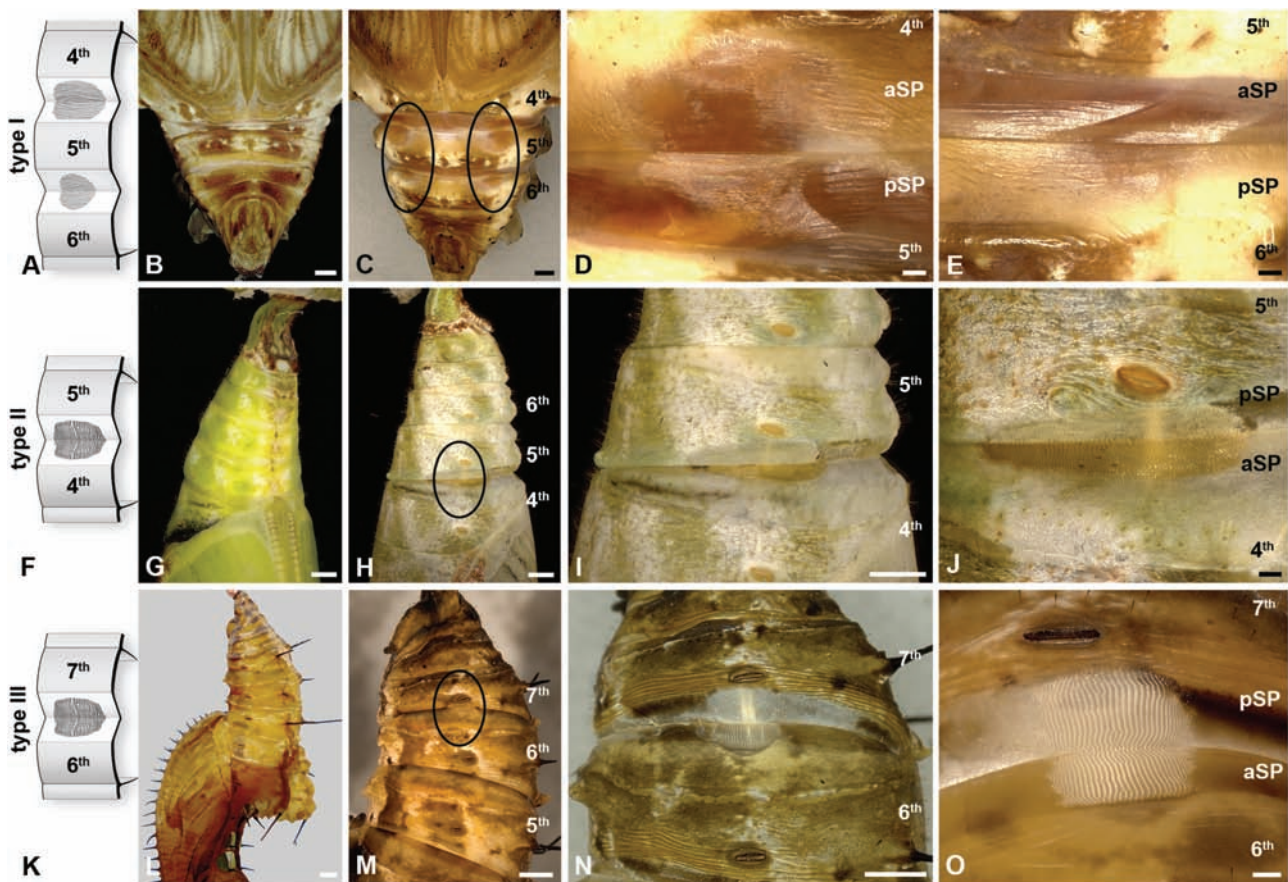


Fig. 3. Pupal SPOs in Papilioninae (type I), Epicaliini (type II) and Heliconiini (type III) exemplified for *Pachliopta kotzebueae* (A–E; viewed ventrally), *Catonephele numilia* (F–J; viewed laterally) and *Heliconius hecale* (K–O; viewed laterally). A, F, K sketches of locations of SPOs on intersegmental membranes between the respective abdominal segments; B, G, L pupae in quiescent position; C, H, M pupae with artificially flexed abdomen, SPOs encircled; D higher magnification of SPO type I between abdominal segments 4/5 and E between 5/6; I, J higher magnification of SPO type II between abdominal segments 4/5; N, O higher magnification of SPO type III between abdominal segments 6/7. Scale bars: B, C, G, H, I, L, M, N = 1 mm; D, E, J, O = 0.2 mm.

their natural buckling (often with additional dents or cracks from drying) makes measurements and counts imprecise. Since the species were selected opportunistically, a comparative interpretation would be not meaningful. Thus, we refrain from providing data on specific differences in the topography of SPOs but keep the focus on general descriptions of the organs.

Sounds

Recordings with the Baton bat detector are sufficient to resolve the basic acoustic properties (i.e., sound production per se, arrangement of twitters in series, number of twitters per series) of pupal sounds in Papilioninae, Epicaliini, and Heliconiini (Fig. 7) but fail when investigating the spectral and temporal fine structure any further. Sound recordings with the Knowles microphone and the custom-made ultrasound recording system allow a detailed sound analysis and also the display of the entire temporal and spectral characteristics of the signals (Fig. 8).

In general, the sounds of pupae can be described as series of acoustically isolated sounds, which we call twitters. Each twitter consists of a series of short pulses (damped oscillations), often separated by pulse intervals (Fig. 8). A series of twitters lasts about 2 s; the number of twitters within a series, the duration of twitters and also the duration of the silent intervals between the twitters (twitter intervals) differ between species (Table 1, Fig. 8A and B). The number of pulses per twitter, pulse duration, and pulse interval differ

between species (Table 1, Fig. 8C and D). The amplitude of the pulses within a twitter varies in a typical pattern with a crescendo at the beginning and decrescendo towards the end of a twitter. The short pulses (0.3–0.8 ms) have a broad spectrum with a considerable portion extending into ultrasound (up to 100 kHz) but the main energy is emitted in the audible range mainly between 3 and 13 kHz (Table 1, Fig. 8B–D). The shape of the pulses is similar for all species, starting with a brief positive phase, followed by an accentuated negative phase, which fade as a frequency modulated oscillation (Fig. 8D). Though pulse patterns were generally not identical but often irregularly distributed within species or individuals, separation of SPOs during wriggling always produced a series of damped pulses quite similar in structure for each species.

Each twitter coincides with a movement of the pupal abdomen and opening of the intersegmental membranes suggesting that sound is produced when SPs separate. Muting pupae with immersion oil demonstrated that the presumed SPOs are indeed responsible for the acoustic emissions. When treating lateral SPOs of Epicaliini or Heliconiini with immersion oil only half of the twitters per series are produced, and the twitter interval approximately doubled in duration (Fig. 9A). If the opposing SPOs were also muted with oil, wriggling did not elicit further twittering. *Papilio lowi* Druce, a species with four ventro-lateral SPOs, did not move voluntarily in the muting experiments though it did in regular sound recordings. However, a twitter could also be elicited by manual flexing the abdomen

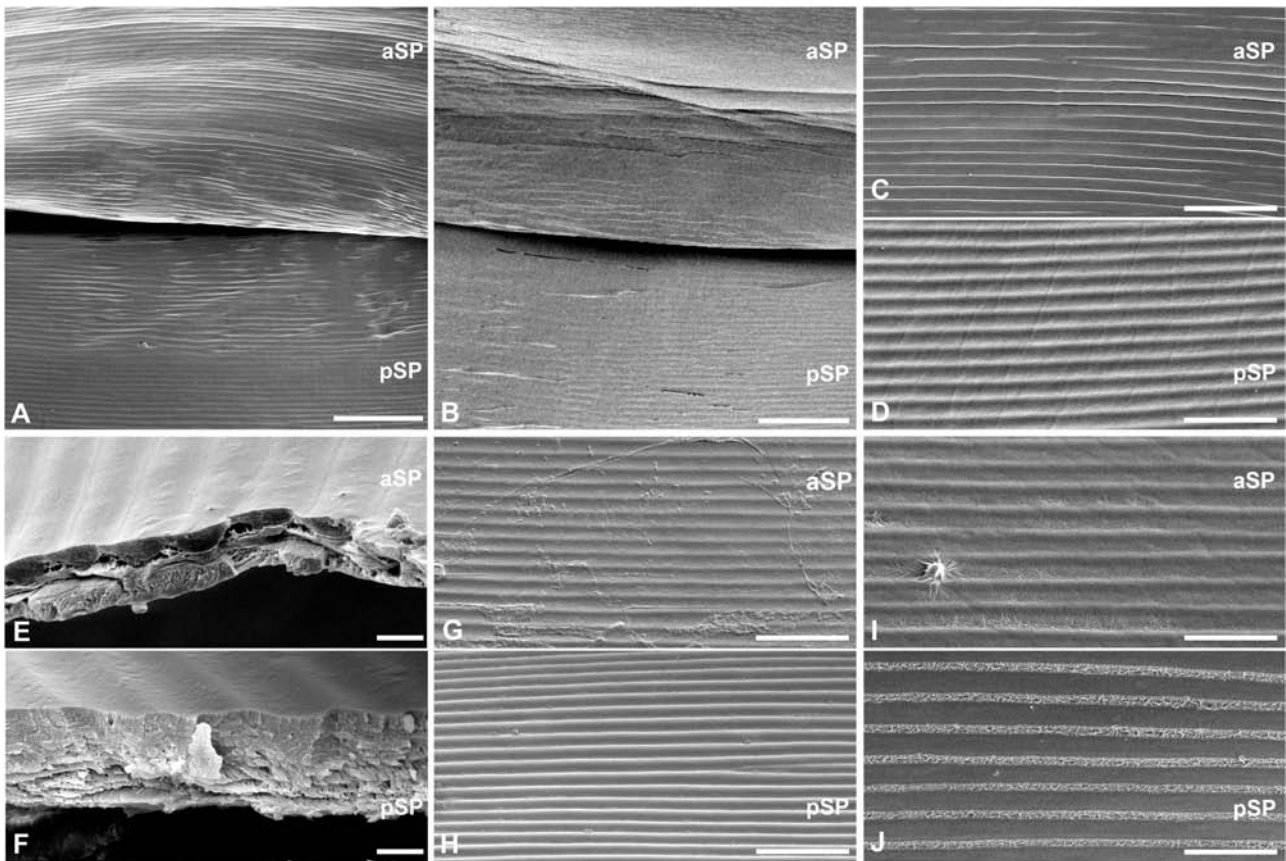


Fig. 4. SEM images of pupal SPOs and anterior and posterior SPs (aSP, pSP) in Papilioninae (type I), *Pachliopta kotzebuea* (A–F), *Papilio polytes* (G, H) and *Parides montezuma* (I, J). Overview of the SPO in *P. kotzebuea* between abdominal segments 4/5 (A) and 5/6 (B); aSP (C) and pSP (D) of 4/5 in higher magnification; fractured aSP (E) and pSP (F) showing multi layers of solid chitin; G, H aSP and pSP of *P. polytes* and I, J of *P. montezuma* showing the same structural elements but differ in the distance of the ridges. Scale bars: A, B = 90 μm ; C, D, G–J = 40 μm ; E, F = 5 μm .

towards ventral (i.e., intersegmental membranes 4/5 and 5/6 open up) in the dorso-ventral axis complying to the voluntary wriggling movements of *P. lowi*. Successive inhibition of the four SPOs with oil revealed a decrease of pulse numbers within the manually produced twitters thus demonstrating that the groups of pulses forming a twitter are produced by the four SPOs between segments 4/5 and 5/6 almost simultaneously (Fig. 9B). Treatment with oil shows that sound production only occurs during separation of aSP and pSP but not while the two reconnect, i.e., the interlocking of the two opposing SPs occurs without any detectable noise (cf. Fig. 9, A2).

In accordance with the morphologically identified types of SPOs, three types of sounds could be discriminated (see Table 1, Fig. 8).

Sounds of type I in Papilionidae: Papilioninae: Troidini (*Pachliopta kotzebuea*, *Battus polydamas*, *Parides montezuma*) consist of series of 2–3 twitters per 2,000 ms (Fig. 8, left column). The twitters are short (14–74 ms) and separated by a long twitter interval (407–1,744 ms). The main energy of the twitters is between 5 and 14 kHz measured at –30 dB from the peak amplitude although there are differences within the papilionid species (Table 1). In *P. kotzebuea*, sound occurs when the SPOs between segments 4/5 separate from each other; supposed SPOs between segments 5/6 appear mute. The number of pulses ranges from 23 to 100 per twitter. No pulse interval could be identified since the pulses within a twitter are irregularly distributed and highly overlap. For *P. kotzebuea*, it was possible to measure pulse duration (0.4 ms) from sequences (10 ms) with a regular distributed pulse pattern although the pulses were not separated by a pulse interval. In contrast, *P. lowi* (Papilionini)

produces twitters with all four organs (between segments 4/5 and 5/6) almost simultaneously.

Sounds of type II in Nymphalidae: Biblidinae: Epicaliini (*Catonephele numilia*, *Nessaea aglaura*) consist of series of 4–5 twitters per 2,000 ms (Fig. 8, center column). The twitters have a duration of 42–129 ms separated by an interval of 53–1,189 ms. The main energy is between 4 and 13 kHz measured at –30 dB from the peak amplitude. Epicaliini emit twitters with the highest amplitude of all pupae recorded but with the lowest bandwidth of all. Twitters of Epicaliini consist of 45–182 pulses per twitter. Within twitters short sequences (10 ms) with clearly separated pulses can be identified. The duration of pulses within these sequences ranges from 0.5 to 0.8 ms with pulse intervals of 0.4–0.6 ms. Estimates of pulse duration and pulse interval for complete twitters were not possible because of low amplitudes at the beginning and end of the twitters and overlapping distributed pulses.

Sounds of type III in Nymphalidae: Heliconiinae: Heliconiini (*Heliconius hecale*, *Dryas iulia*) consist of series of 8–15 twitters per 2,000 ms (Fig. 8, right column). The twitters are short in duration (15–125 ms) separated through a short interval (24–229 ms). The relative position of the SPOs of type II and III and their similar wriggling movement results in a similar acoustic pattern. The twitters are arranged in groups but in contrast to the Epicaliini with a higher total number of twitters grouped together (cf. Fig. 8A). The position of SPOs on a pupa has no influence on basic acoustic patterns. Within the twitters of Heliconiini each pulse is distinct—clearly separated from each other—but with a very similar pulse structure

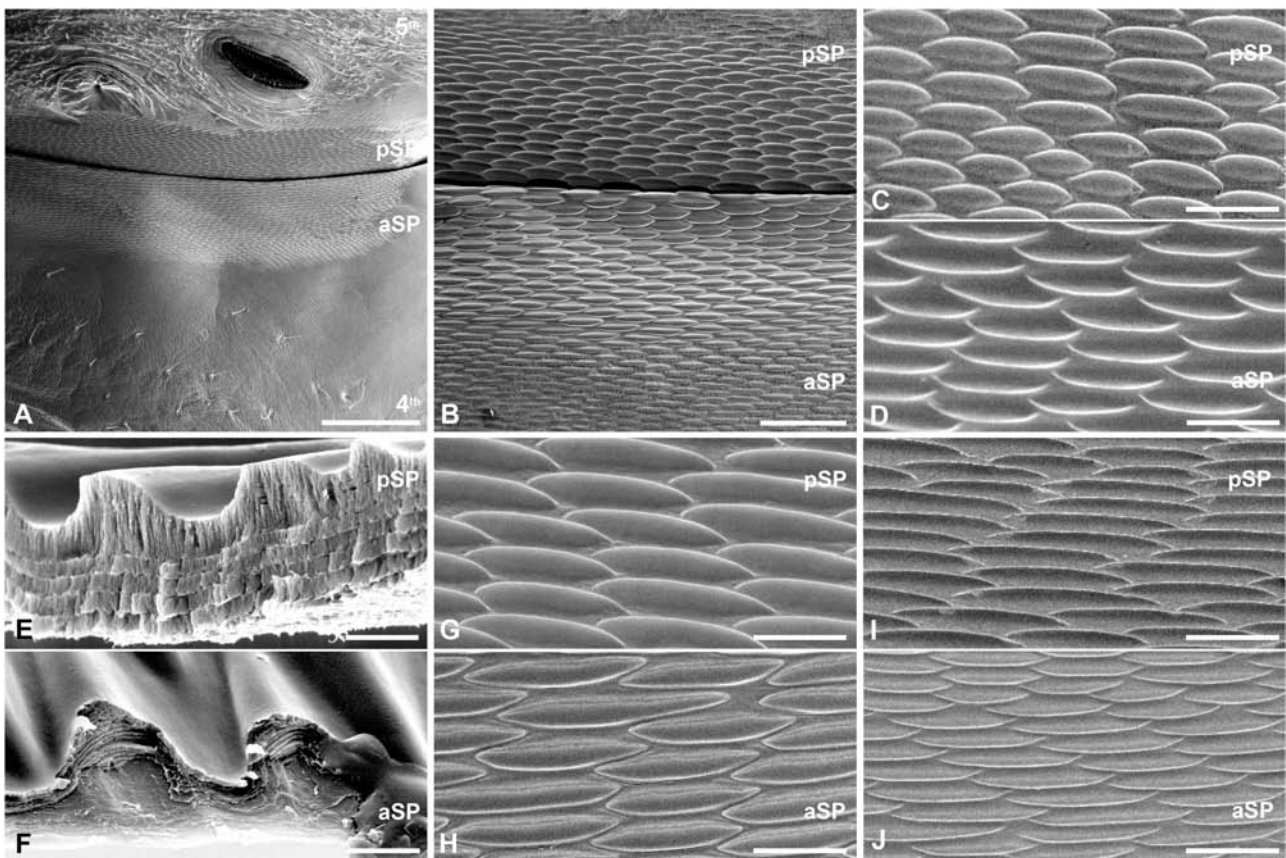


Fig. 5. SEM images of pupal SPOs and anterior and posterior SPs (aSP, pSP) in Epicaliini (type II), *Catonephele numilia* (A–F), *Nessaea aglaura* (G, H) and *Myscelia cyaniris* (I, J). Overview of the SPO between abdominal segments 4/5 (A); pSP and aSP in higher magnification (B–D); fractured pSP (E) and aSP (F); G, H pSP and aSP of *N. aglaura* and I, J of *M. cyaniris* showing similar microstructures but size differences. Scale bars: A = 400 μm; B = 90 μm; C, D, G–J = 40 μm; E, F = 5 μm.

(Fig. 8C). The main energy of a twitter is 4–21 kHz measured at –30 dB from the peak amplitude. In comparison to the sounds of Troidini and Epicaliini the amplitude of the twitters is very low but the signal can reach 126 kHz measured at –30 dB from peak amplitude in *H. hecale*. In *Heliconius*, series often start with one or two twitters extended in duration in comparison to the subsequent twitters of the series (Figs 7C and 8A). Twitters of *Heliconiini* consisted of 25–245 pulses per twitter. As in Epicaliini, sequences (10 ms) with clearly separated pulses and pulse intervals were identified. The duration of pulses in *H. hecale* is 0.3 ms with pulse intervals of 0.6 ms; complete measurements of pulse duration per twitter were not possible.

An audio file of the twitter series of *P. kotzebuea*, *C. numila*, and *H. hecale* presented in Figure 8 is provided (Supp. Appendix 1 [online only]).

Discussion

We should emphasize that our selection of species for investigation is heavily biased. It is very likely that SPOs will be found in pupae of many more taxa of Papilionoidea (cf. Fig. 1), although Downey (1966) found no stridulating devices in pupae of several Nearctic Papilionidae and Nymphalidae.

Type I in our study is basically the one which Hinton (1969) had briefly described for *Troides*. While he found sound producing structures in the intersegmental membrane between segments 4/5 only, in other Troidini (*Battus*, *Parides*) we found SPOs between 4/5 and 5/6. Did Hinton (1969) overlook the second organ in *Troides*? Type

II and III are basically the same, however, the SPOs are located on different segments. It remains a surprise that in Nymphalidae SPOs were found between segments 4/5 or 6/7 only but (yet?) between 5/6; in Lycaenidae pupae stridulatory structures occur between all the abdominal segments in question (Downey 1966; Downey and Allyn 1973; Pierce et al. 2002). Unlike the Lycaenidae, where sound organs may completely encircle the pupa (Downey 1966), in the Papilionidae and Nymphalidae the SPOs are distinct paired organs, the rest of the intersegmental membranes being unmodified.

When comparing papilionid and nymphalid pupae we basically see two types of SPOs, one with ridges, one with ± oval elevations or depressions. We did not survey the microstructures in every detail and thus cannot comment on species-specificity of characters or correlate, e.g., duration of the twitters to the size of the SPOs. Due to the nature of the preparations (see above), statistically meaningful data cannot be generated but novel structures and sounds can be characterized.

Comparisons between living pupae and exuviae revealed no obvious structural differences. Thus, exuviae are well-suited for morphological investigations. However, for characterizing the sculpturing of the SPOs, histological studies are needed (see Fig. 10B) because transverse fracturing of dry exuviae—as we did—is largely uncontrollable and does not give clear results (Figs 4E and F, 5E and F, 6E and F).

Eliciting Pupal Movement

We stimulated pupae manually or by brushing. Possibly we could have elicited more natural movements if we had been aware of

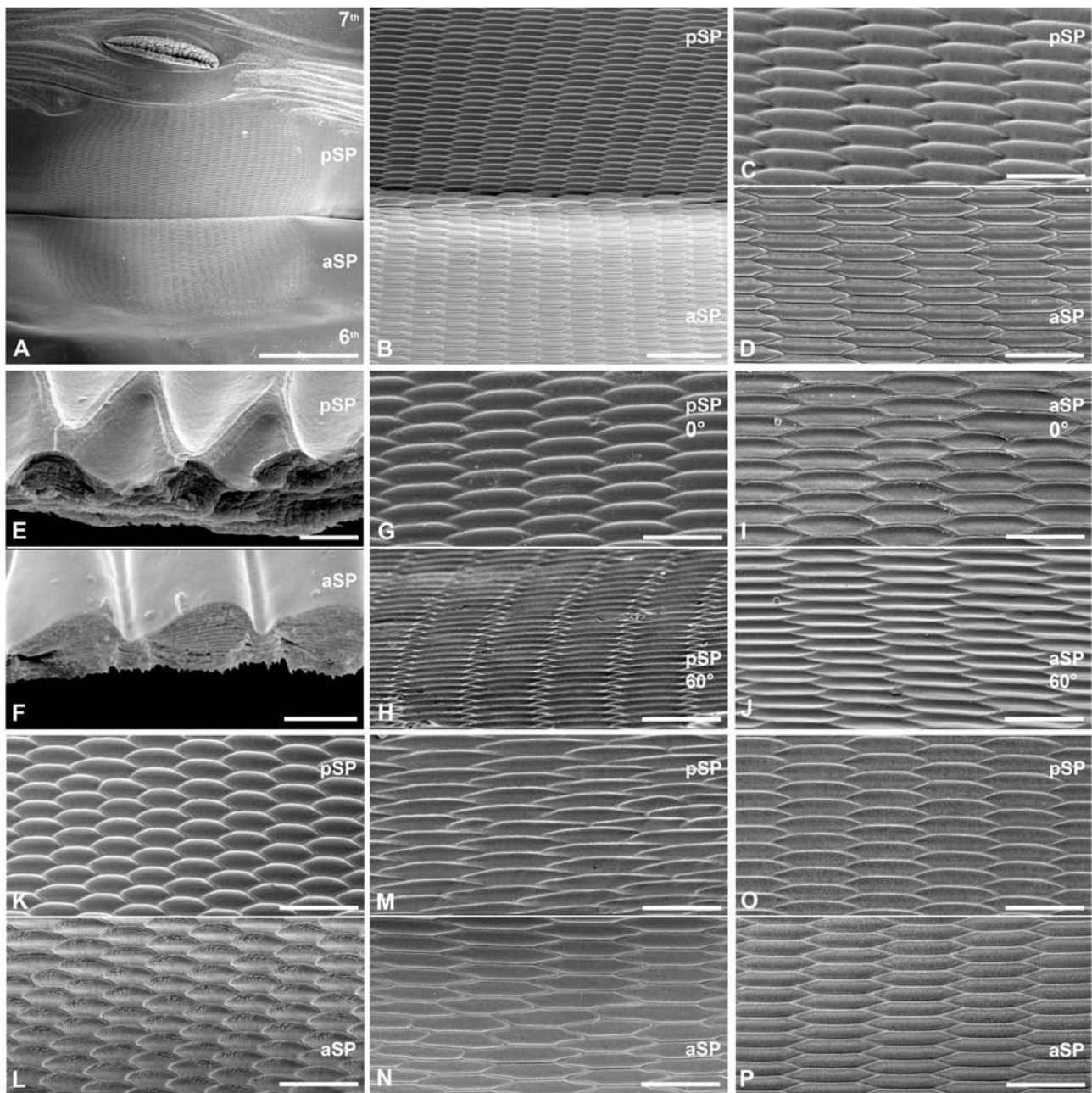


Fig. 6. SEM images of pupal SPOs and anterior and posterior SPs (aSP, pSP) in Heliconiini (type III), *Heliconius hecale* (A–J), *Philaethria dido* (K, L), *Laporus doris* (M, N), and *Dryas iulia* (O, P). Overview of the SPO between abdominal segments 6/7 (A); pSP and aSP in higher magnification (B–D), fractured pSP (E) and aSP (F); G–J SPs in different viewing angles show different appearance of the surface: pSP (G, H) and aSP (I, J) in 0° and 60°, respectively. K, L pSP and aSP of *P. dido*, M, N of *L. doris*, and O, P *D. iulia* show the same microstructures but differ in size and shape. Scale bars: A = 400 μm; B = 90 μm; C, D, G–P = 40 μm; E, F = 5 μm.

Cole (1959): pupae of the nymphalid *Aglais urticae* (Linnaeus) vary in their movement responses, with only few sluggish movements when stroked with a hair brush but with violent wriggling when touched by the antenna or tarsus of an Ichneumonid wasp. Thus, very local (punctual) excitation might be better than stimulation of wider areas.

We were not able to identify sensitive structures (e.g., sensilla) responsible for eliciting the wriggle response in pupae. However, pupal abdomina exhibit many hairs (Fig. 11) which might well represent sensilla—little is known about the sensory structures of the pupal cuticle in Lepidoptera (Linden 1902). Perhaps, there are parallels to the gin traps described by Hinton (1955) as a defense

in lepidopteran and coleopteran pupae. In *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), the gin traps are located between the abdominal segments 1–7 (Wilson 1971) and in *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) between segments 4–7 (Bate 1973). The traps, which function as a mechanical device against antagonists (Bate 1973; Eisner and Eisner 1992), may have evolved into a sound producing apparatus in pupae of *Allomyrina dichotoma* Linnaeus (as *Trypoxylus*) (Coleoptera: Scarabaeidae: Dynastinae) (Kojima et al. 2012); pupal movement is triggered by mechanosensory hairs within the gin traps (Bate 1973; Kurauchi et al. 2011).

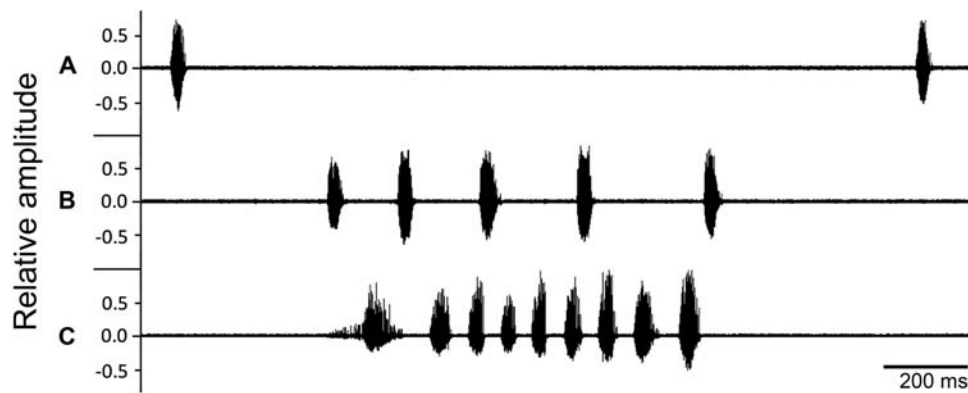


Fig. 7. Series of representative twitters produced by *Papilio thoas* (Papilionini; A), *Myscelia cyaniris* (Epicaliini; B) and *Heliconius melpomene* (Heliconiini; C) recorded with the Baton bat detector and displayed with Audacity 2.0.6. The phase information is incomplete but the recordings principally document the sound production of pupae and their time course.

Sound Generation

Typical stridulatory organs in insects consist of a file and a scraper, i.e., morphologically dissimilar structures which are rubbed together to produce frictional sounds (Prell 1913; Dumortier 1963). In the context of butterfly pupae, the organs of the Lycaenidae and Riodinidae seem to match this mechanism (Prell 1913; Downey 1966). Apart from basic structural differences of the sound producing apparatus, there are acoustic similarities and dissimilarities between lycaenid pupae and pupae investigated in our study. The sounds of Lycaenidae are lower in frequency, ranging between 0.1 and 4.5 kHz with main energies notably around 0.5–1 kHz (Barbero et al. 2009; Álvarez et al. 2018), thus far below the frequencies we have measured. However the temporal structure of sounds of lycaenid pupae (Hoegh-Guldberg 1972; Álvarez et al. 2018) is similar to the twitters we describe. The very clear examples for typical stridulation include the distress sounds produced by adult velvet ants (Hymenoptera: Mutillidae) (Masters 1979, 1980; Polidori et al. 2013) which stridulate in the classic sense by rubbing a scraper located at the posterior margin of tergite II over a stridulatory file located medially on the anterior portion of tergite III (Polidori et al. 2013). While SPOs of type I (Papilioninae) pupae and mutillid stridulatory files seem almost identical structures (regular, transverse ridges, or striations; Polidori et al. 2013), the microstructures of the latter are brought into oscillation through a scraper moving over files (Tschuch and Brothers 1999). When stridulating, velvet ants produce chirps generally structured into two syllables, which originate in the forward and backward movement of the metasomal scraper over the file (Polidori et al. 2013). In pupal sounds of Nymphalidae and Papilionidae, the corresponding second acoustic syllable (the backward movement) is obviously missing. This has been confirmed by muting the SPOs and checking the manipulated pupae for sound production (Fig. 9). As shown above, only when the SPs of nymphalid and papilionid pupae separate from each other, sound is emitted.

In contrast to pupae of lycaenids and to mutillids, in pupae of Papilionidae and Nymphalidae the opposing structures of the sound organs, the aSPs and pSPs, are generally very similar, representing elevations and depressions, one appearing as an imprint of the other. Sealy (1875) stated ‘... I thought at first it was merely produced by the rubbing of one ring of the pupa case against the next, but the sound did not resemble a mere frictional sound, it was more like the sound of a rush of air through small holes—“pha, pha!”’, while Hinton (1969) suggested for *Troides* pupae that the ‘ridges of one half of the organ fit into the grooves between the ridges of the other half ... sound is produced by collisions as the ridges are pulled out of

the grooves ... The process is analogous to unzipping a zip-fastener’. Our data support Hinton’s (1969) view: papilionid as well as nymphalid pupae do not produce frictional sounds in the classic sense by stridulation of two different structures (scraper and file causing the picket-fence or washboard effect; see also Akay 2002) but rather by pulling apart mirror-image microstructures which at rest are interlocked (consider the two opposed parts making up poppers; see Fig. 9); when the structures interlock, no sounds are generated.

Thus, sound generation by friction through stridulation needs to be differentiated from sound generation by friction through pulling apart, and for pupal sounds in papilionid and nymphalid butterflies we suggest a stick-slip mechanism sensu Akay (2002) although we do not know of another such case in insects. To highlight the distinction to stridulation we introduce the term ‘twitter’ to describe the acoustics emitted by papilionid and nymphalid pupae although ‘calls’ (Travassos and Pierce 2000; Pierce et al. 2002) or ‘pulse-trains’ (Hoegh-Guldberg 1972), used for lycaenid emissions, seem equivalent.

In studies on the physics of friction in microstructures, similarities to the pupal twittering can be recognized. Stick-slip motion is defined as ‘the quasi-periodic “sticking” and “slipping” of contacting surfaces under relative motion’ (Baum et al. 2014) and can occur at a macroscopic to an atomic level (for details on the physics of sliding friction see Persson 2000). The absence of a scraper-like structure in our butterfly pupae suggests that there is static friction (sticking force) between two opposing and interlocking SPOs which even increases with the outward pulling if the pupa is moving; it could also be denoted as adhesive stick-slip mechanism (B. N. J. Persson, personal communication). When at a specific stage the kinetic friction (sliding force) exceeds the sticking friction, the two SPOs slide apart relative to each other. Hence, e.g., in Papilioninae, when a ridge slides away from its mirror image, a single pulse is generated and—as all ridges are pulled out successively—a twitter sound consisting of succeeding short pulses is generated.

Baum et al. (2014) have built a model based on the frictional properties of scales of the snake *Lampropeltis getula californica* (Blainville) (Squamata: Colubridae) to investigate friction behavior of microstructured polymers, which presents a surprisingly detailed analogy to the SPOs of Papilioninae. In their snake-inspired model, where a microstructured surface is moved *against* its counterpart (corresponding to the pulling apart of SPs) higher amplitudes are produced than when moved *along* the microstructure (corresponding the interlocking of aSP and pSP) and lower amplitudes are emitted.

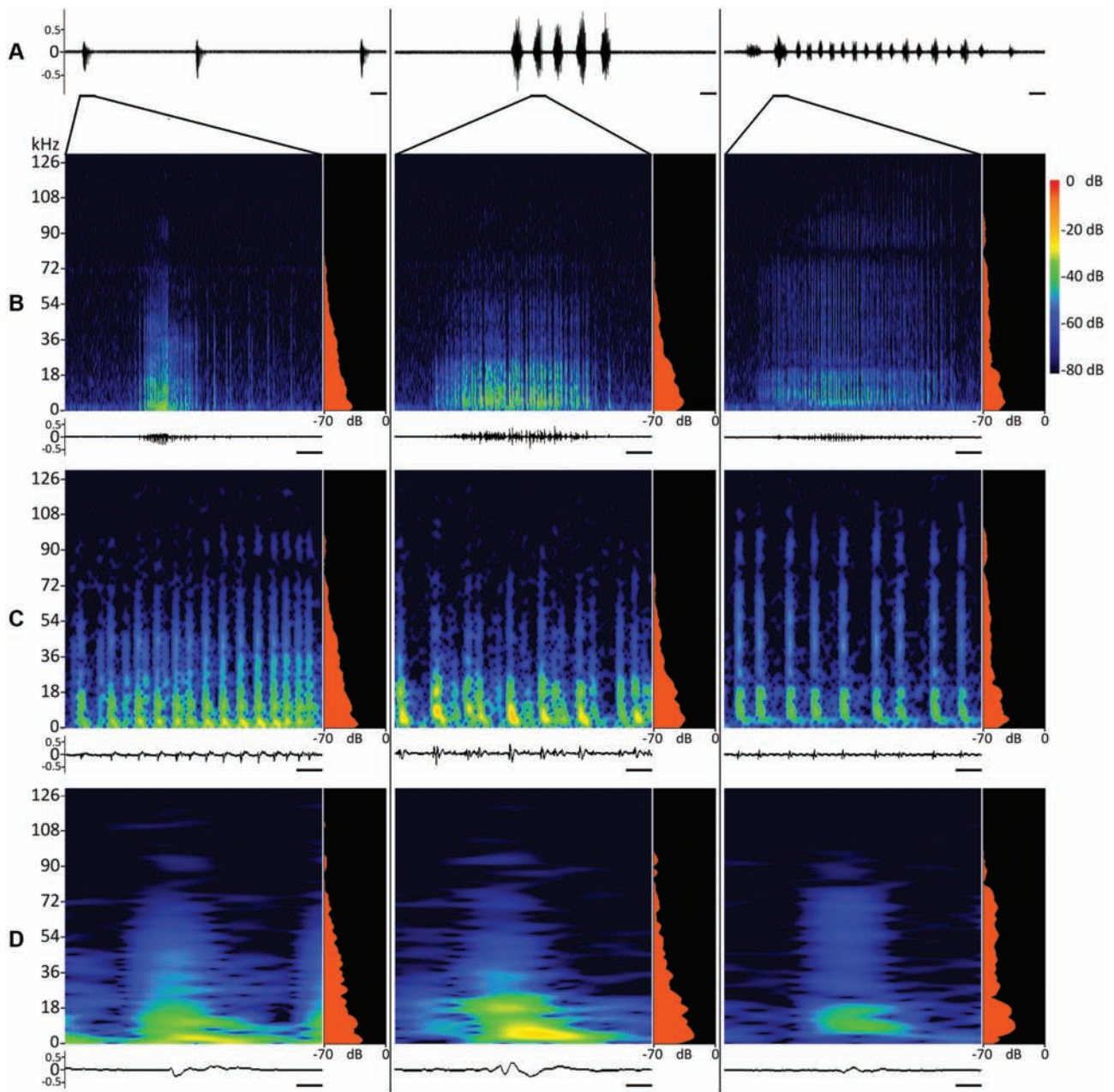


Fig. 8. Representative twitters produced by *Pachliopta kotzebuea* (Troidini; left), *Catonephele numilia* (Epicaliini; center) and *Heliconius hecale* (Heliconiini; right). The upper row (A) illustrates temporal patterns in series of pupal twitters with Papilioninae producing 2–3, Epicaliini 3–5 and Heliconiini 8–15 twitters per 2,000 ms. The acoustic fine structure displayed as color spectrograms, power spectra and oscillograms at different time sections (A–D) reveals that each twitter is composed of a more or less regular series of short pulses (damped oscillations; B–D). While sounds of Papilioninae (left column) can be characterized as twitter series with long twitter intervals, missing pulse intervals, pulse overlaps and irregular distributed pulse patterns, Epicaliini (central column) and Heliconiini (right column) produce series of clearly grouped twitters, which often possess more regular pulse distributions with distinct pulse intervals. Scale bars: A = 100 ms; B = 10 ms; C = 1 ms; D = 0.1 ms. An audio file of the twitters shown is provided online in the supporting information.

The only arthropods so far known to use a stick-slip mechanism to produce acoustic emissions are spiny lobsters (Palinuridae; Patek 2001). They rub two macroscopically smooth surfaces together, causing microstructural surfaces to periodically induce stick-slip events (Patek 2001; Patek and Baio 2007) which result in a pulsed sound (Patek 2001) (only) during the sliding movement of one surface over the other (Patek 2002). Spiny lobsters produce these sounds when disturbed indicating a defensive function of the acoustic emissions (Staaterman et al. 2009). Remarkably, the stick-slip mechanism in spiny lobsters seems independent from the hardness of a scraper

or a file, thus being theoretically functional even in critical periods when the lobsters moult (Patek 2001)—also a parallel to the butterfly pupae? Advanced approaches, e.g., high-speed video recordings or enlarged SPO replicates, might provide further insights on how exactly sound is produced in twittering pupae.

Recording Devices

With a Baton bat detector detailed temporal and spectral analyses are not possible because part of the phase information of the temporal signal is lost (Fig. 7) and signal processing during recording

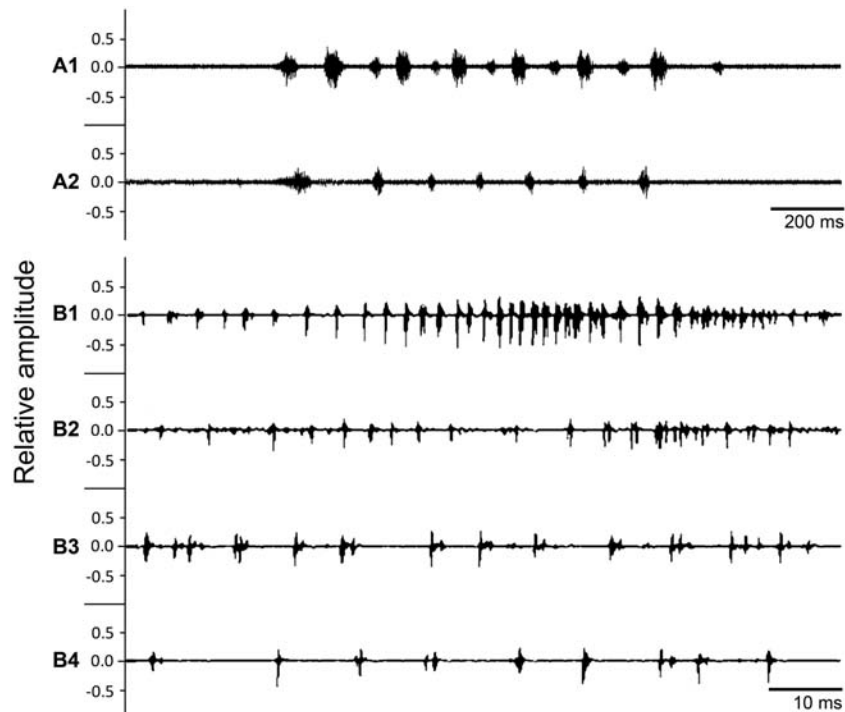


Fig. 9. Muting of SPOs in nymphalid (A) and papilionid (B) pupae. *Heliconius hecale* (A) produces sounds through lateral movements with two SPOs acting alternately (A1). If one of the two lateral SPOs is treated with immersion oil sound is only emitted when the SPs of the untreated side separate (A2). The acoustic properties remain the same but every second twitter is missing. *Papilio lowi* (B) on the other hand produces sounds through dorso-ventral movements with four ventro-lateral SPOs acting simultaneously. Manual flexing of a pupa with unmanipulated SPOs (B1) and progressive muting of all four SPOs between segments 4/5 right side (B2), subsequent of SPO 4/5 left side (B3) and of SPO 5/6 right side (B4) shows that pulse number decreases with each disabled SPO. Differences in amplitude are possibly caused by differences in the strength by which the pupa was flexed.

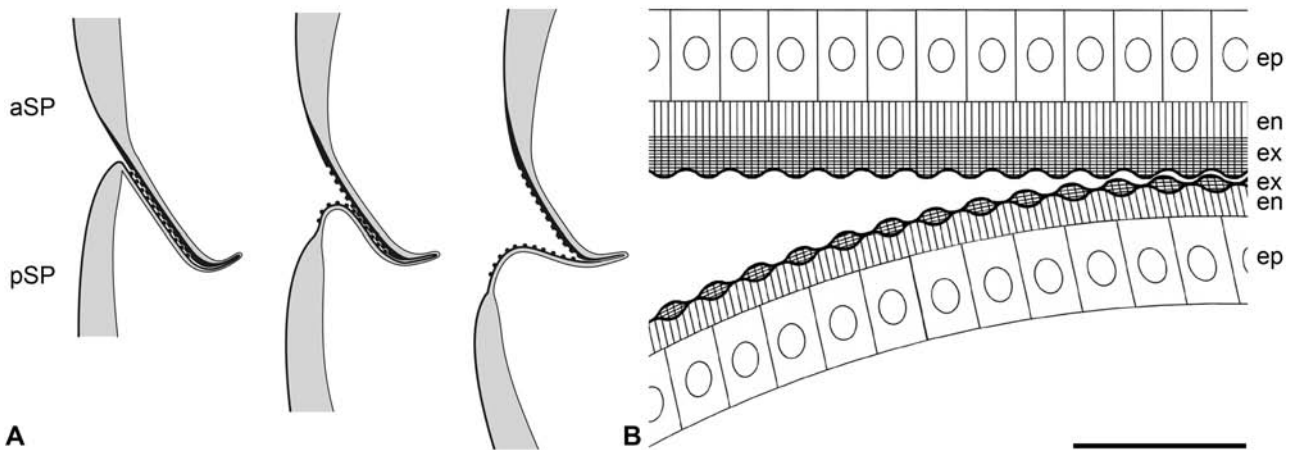


Fig. 10. Sketches illustrating the process of pulling apart the SPs during pupal wriggling (A) and the integument of SPs (B; true to scale; after histological semi-thin cross sections of *Heliconius hecale* (Fischer, Kiesel, Dolle unpubl.)) in the course of separating the posterior (pSP) from the anterior (aSP) SP which generates sounds, not by classic stridulation but likely by stick-slip friction. In pSPs the exocuticle (ex) is thinner than in aSPs and thus more flexible, permitting pulling the pSP apart from the more solid aSP during wriggling. ep epidermis, en endocuticle. Scale bar: 50 μ m.

is incomprehensible. For a detailed sound recording and analysis of pupal twitters, it is necessary to use a recording system with an ultrasonic microphone and a reasonable frequency response between 1 and 100 kHz, an A/D converter with a sampling rate above 200 kHz, and a corresponding sound analysis system. Nevertheless for demonstrating sound production to visitors at a butterfly house or to students and for field studies in the tropics, devices such as the Baton bat detector or even a mobile phone with a most limited sample rate of 44.1 kHz (in combination with open source acoustic software) are

sufficient to obtain adequate information about presence of twitters and time course of twitters and thus prove to be valuable tools.

Sound Characters

The comparison of Troidini, Epicaliini, and Heliconiini reveals that the twitters of type I (Troidini) are emitted at a lower rate with rather long twitter intervals in between, whereas the type II and III twitters of Epicaliini and Heliconiini are grouped in distinct series consisting

of more closely spaced 4–5 twitters in Epicaliini and around 14–15 twitters in *H. hecale*. These patterns reflect the general differences between the movements of girdled versus suspended pupae. The latter are fixed on a surface only at a single point and can exploit the kinetic energy stored in the lateral movement of the pupa so that they produce more succeeding twitters with less energy input. However, this may also be responsible for the temporally more variable twitters of Epicaliini and Heliconiini in comparison to twitters of papilionid pupae (Table 1).

To stimulate wriggling, we held the living pupa manually on the thorax in front of the microphone relying on the experience that this method has no influence on pupal sound production per se. Our finding that number, duration of twitters, and twitter intervals differ between the three types might be artificial and needs further confirmation through recordings of pupae still fixated at their pupation sites. However, the structure and composition of pulses and pulse patterns is determined by the morphology of SPOs and can be used as a constant character to describe differences between the three types of SPOs found. The arrangement of pulses in the type I twitters of

Troidini is more irregular and may reflect an overlap in the parallel activation of two or four ventro-lateral SPOs, respectively. These are positioned side by side on two intersegmental membranes so that when the pupa is moving back and forth two organs are always activated at the same time when the intersegmental membranes open. Since opening of segments 4/5 and 5/6 is concurrent, sound is produced by four almost simultaneously acting SPOs. Within the twitters no pulse intervals could be identified but only overlapping pulse groups. The type II and III twitters of Epicaliini and Heliconiini show a more regular pattern and partially the twitters can be characterized as groups of non-overlapping distinct short pulses which are produced when lateral SPOs open. Some pulses possess a clearly identifiable pulse interval though it is difficult to measure for complete twitters due to change of amplitude and temporal dynamics within twitters. Although we expected pulse numbers to be higher in papilionid pupae (4 SPOs acting almost simultaneously) our estimates show that they are similar or even lower in total number (Table 1)—perhaps, the quite irregular pulse patterns and overlapping pulses seen in Troidini cause errors in counting pulse numbers.

The location of SPOs between segments 4/5 in Epicaliini and 6/7 in Heliconiini can be used as a morphological character to differentiate between type II and III of SPOs but has no influence on the acoustic properties of sounds. Heliconiini differ from Epicaliini insofar as they produce twitters with lower amplitudes and this might be related to the morphological finding that Heliconiini possess the smallest organs. Pulse duration is elongated in Epicaliini, which might turn out to relate to microstructural details of SPs which are yet unstudied.

The comparison of distinct pulses in twitters of nymphalid to overlapping pulses of papilionid pupae might be directly related to the morphological differences of transverse and regular ridges in Papilioninae (Fig. 4) versus the boat-shaped/oval depressions and elevations in Nymphalidae (Figs 5 and 6). When separating pSPs from aSPs in Papilioninae, the edges of these ridges likely scratch against each other, thus produce additional noise through friction and cause the pulsed twitters to become more blurred. On the other hand, the oval depressions and elevations of SPs in Nymphalidae seem to fit each other perfectly, with the effect that separation of the two SPs is free of, or accompanied by little additional noise. Why pulses produced by a complex pattern of depressions and elevations are more distinct than those produced by quite simple ridges remains an open question.

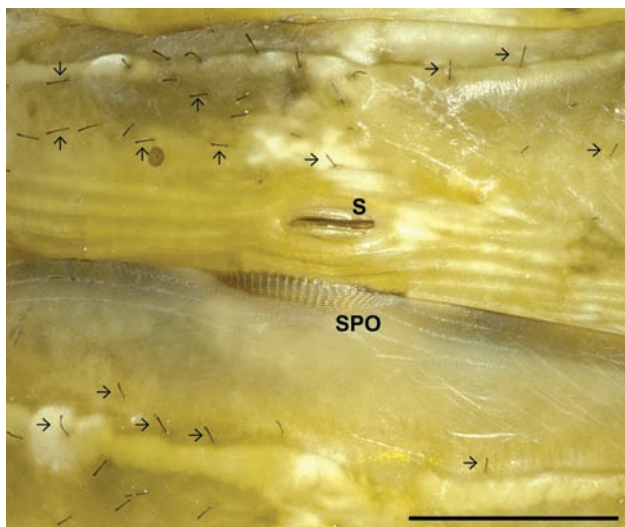


Fig. 11. Pupal sound producing organ (SPO) of *Heliconius hecale* showing adjacent hairs (arrows) which might represent sensilla. S spiracle. Scale bar: 1 mm.

Table 1. Temporal and spectral characteristics of twittering in pupae of seven butterfly species

Species	SPO type	1 no. of twitters/series	2 twitter duration (ms)	3 twitter interval (ms)	4 no. of pulses/twitter	5 pulse duration (ms)	6 pulse interval (ms)	7 main energy (kHz)
<i>Pachliopta kotzebuea</i>	I	2.5 ± 0.6	46.7 ± 16.2	749.7 ± 323.4	81.5 ± 14.4 (62–98)	0.37 ± 0.3	–	3.4 ± 2.1
<i>Battus polydamas</i>	I	2 ± 0	16.4 ± 2.2	541.1 ± 0	33 ± 10.4 (23–47)	–	–	12.7 ± 3.1
<i>Parides montezuma</i>	I	3.2 ± 0.5	20.1 ± 3.4	516.1 ± 93.2	57.8 ± 22.6 (31–100)	–	–	6.9 ± 3.1
<i>Catonephele numilia</i>	II	4.9 ± 1.2	60.6 ± 8.4	90.4 ± 51.6	112.2 ± 38.7 (45–182)	0.45 ± 0.2	0.56 ± 0.4	8.7 ± 2
<i>Nessaia aglaura</i>	II	4 ± 1.6	72.9 ± 21.4	188.4 ± 334.1	88.4 ± 26.7 (54–141)	0.78 ± 0.2	0.44 ± 0.3	9.7 ± 2.8
<i>Dryas iulia</i>	III	8 ± 0	29.4 ± 4.9	115 ± 48.8	53.4 ± 17.2 (28–83)	–	–	6.1 ± 1.1
<i>Heliconius hecale</i>	III	14.6 ± 3.1	40 ± 17.8	59.3 ± 21.5	82.1 ± 40.1 (25–245)	0.3 ± 0.1	0.63 ± 0.4	12.5 ± 4.2

Values are mean ± standard deviation; measurements 1–3 and 7 were taken on 10 twitter series, 4 on 3 twitter series, 5–6 on 3 twitter sequences of 10 ms. For *B. polydamas* and *D. Iulia*, only one twitter series was available.

SPO type: see text; no. of twitters/series: number of twitters produced within 2,000 ms; twitter duration: duration from the first to the last deflection of a twitter of the oscillogram; twitter interval: silent interval between twitters within a series; no. of pulses/twitter: estimated number of pulses (min–max); pulse interval: silent interval between subsequent pulses; main energy: frequency with the highest amplitude; –: no measurements possible (see text).

Functional Aspects of Pupal Sounds

Pupae are confronted with a wide range of antagonists and have developed a broad spectrum of passive and active protective devices (for reviews, see [Hinton 1948, 1955](#)); for a general overview of important parasitoids and predators of the various life stages (including pupae) of neotropical butterflies, see [DeVries \(1987\)](#). With respect to functions of the sounds emitted by the pupae we investigated, hardly any other role seems likely than defense against antagonists. Broadband calls generally fall into the hearing range of a variety of vertebrate and invertebrate predators (for a comparative compilation of hearing sensitivity ranges, see [Sales and Pye \(1974\)](#); for more detailed information and further references, see [Köppel and Manley \(2014\)](#)). Reviewing defensive mechanisms in lepidopterous pupae against parasitoids [Cole \(1959\)](#) did not mention sounds; he found violent wriggling alone to be sufficient for a pupa to shake off parasitoids.

To avoid the attack of a hearing predator, it is plausible that sounds reinforce a startle response caused by wriggling of pupae upon disturbance. As [Masters \(1979\)](#) pointed out, besides a startle function, the other mode of disturbance sounds might be to inform the recipient of, for example, unpalatability which is likely for at least some species ([Nahrstedt and Davis 1983](#)).

However, in several Lycaenidae, the sounds are highly specific and serve a role in the interaction with ant hosts ([Downey 1966](#); [Efferich 1988](#); [Travassos and Pierce 2000](#); [Pierce et al. 2002](#)), and in those Lycaenidae ([Elgar and Pierce 1988](#)) and Heliconiinae ([Brower 1997](#) and references therein) which perform pupal mating, sounds might serve sex recognition (see [Downey 1966](#); [Hay-Roe 2006](#)). It is worth noting here that the function of lycaenid and riodinid pupal sound is still under debate because there are non-ant-tended species known to produce sounds ([Álvarez et al. 2014, 2018](#)). For beetles, it has been discussed that pupal sounds might serve the purpose of local congregation of larvae ([Prell 1913](#)) or to deter conspecifics from aggregation ([Kojima et al. 2012](#)).

Varieties of insects have evolved acoustic antipredator devices and defend themselves through their different life stages with diverse SPOs ([Alexander 1957](#); [Masters 1979](#); [Bura et al. 2011](#); [Conner and Corcoran 2012](#)). Examples include *Amorpha juglandis* larvae (J.E. Smith) (Lepidoptera: Sphingidae) ([Bura et al. 2011](#)), velvet ants (Hymenoptera: Mutillidae) ([Masters 1979](#); [Polidori et al. 2013](#)) and the hawkmoth *Xylophanes falco* (Walker) (Lepidoptera: Sphingidae) ([Kawahara and Barber 2015](#))—all of these have been shown in feeding experiments to reduce attack rates of potential predators through acoustic emissions.

The question against which antagonists butterfly pupae need to defend themselves and against which predators or parasitoids the observed sounds are effective deterring signals is challenging and remains widely open. However, the structures of SPOs documented here suggest a high degree of adaptation—but to which selecting agent?

We assume that movement as well as broadband twitters produced by Papilioninae, Epicaliini, and Heliconiini pupae *a priori* serve a defensive function, designed for the perception of recipients capable of hearing airborne sounds up to 100 kHz. We were unable to specify at which distances the pupal sounds can be detected since we had no calibrated recording system. At a distance of 1–2 cm, the pupal sounds are clearly audible for younger persons. With increasing distance the twitterings become more and more faint, not detectable for humans at about 30 cm. There might be a direct link between broadband twitters and pupal attachment substrate (e.g., leaf, twig, etc) since at least in vibrational communication systems broadband signals are better suited for communication through

plants than narrowband signals ([Michelsen et al. 1982](#)). It needs to be considered that movement/sound production is elicited by direct contact or, perhaps, airborne stimulation of pupal mechanoreceptors, and therefore an elicitor (= recipient?) must necessarily be very close to a pupa if not touching it.

Perspectives

Our limited study is focused on general characterization of SPOs and sounds in non-lycaenid butterfly pupae, and opens a rich field for further studies, interesting for a variety of disciplines. Very likely, the distribution of SPOs in Papilionidae and Nymphalidae will turn out not to be so ‘erratic’ as one might assume from this study. The many questions remaining include: Which are natural elicitors for pupal sound production? Which are the selective agents acting on these organs? Which are the sensitive parts of the pupae relevant for eliciting wriggling? How and when are the elaborate microstructures formed, in prepupae or in the course of moulting? Are there species exhibiting species-specificity and/or sex-dimorphism in their twitters? What about adaption of the behavioral response to mechanical stimulation? How can the frictional mechanics of a stick-slip sound production be characterized? Is there intra- or interspecific and/or age variation? Species of *Ornithoptera* and *Troides* (see [Matsuka 2001](#)) because of their large size might be particularly handy study objects.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

Acknowledgments

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