

Reproductive biology and embryonic development of *Nocticola* sp. (Blattodea: Nocticolidae)

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Abstract. Reproductive biology, including the mating behavior, ootheca rotation and deposition, and the embryonic development of a nocticolid cockroach *Nocticola* sp. were examined and described, and the phylogenetic issues regarding Nocticolidae and Blattodea were discussed. The abdominal terga II to IV are specialized in males, relating to the tergal glands, although they are less specialized in macropterous males. The (brachypterous) males display a wing-flapping behavior around the female before mating. The female delivers the ootheca with its keel upward, and several hours later, rotates it clockwise by 90° viewed from the female's side, into a horizontal position. After carrying the ootheca for a few days holding it by its flange, the female deposits it on the ground. The ootheca is pear-shaped, and contains only four eggs. About a dozen micropyles are localized on the posterior region of the ventral side of the egg. The aggregation of symbiotic bacteria (“mycetome”) is not detected in the eggs. The embryo undergoes the blastokinesis of the “non-reversion type,” maintaining the original orientation in the egg. Similar to the recent combined and phylogenomic analyses, the information on the reproductive biology obtained in the present study, including the manner of handling of ootheca, mode of mating behavior, egg structure, and type of blastokinesis, may suggest close affinity between Nocticolidae and Corydiidae, thus the monophyletic Corydioidea.

Key words. Blattodea, Nocticolidae, reproductive biology, mating behavior, ootheca, egg, embryonic development.

1. Introduction

The small blattodean family Nocticolidae comprises small, delicate insects. Their bodies are slender rather than dorsoventrally flattened like that of other cockroaches, and their antennae and legs are elongated and attenuated. In males with well-developed wings, the fore and hind wings are similar, being membranous, hyaline, and having few veins, but the wings are reduced to a varied extent. Females are apterous except for *Phleossilpha cavicola* (Chopard, 1958), *Spelaeoblatta gestroi* Bolívar, 1897, *Spelaeoblatta thamfaranga* Roth, 1994, and *Spelaeoblatta myugei* Vidlička, Vršanský & Shcherbakov, 2003. Macropterous males have well-developed compound eyes, but compound eyes are absent or reduced in brachypterous males and females. Arolia and pulvilli are absent. Ten genera and 38 species have been described in Nocticolidae (cf. GUPTA & CHANDRA 2019; SENDI et al. 2020). Most of the species are cavernicolous, a few are epigeal or termitophilous,

and several can be found both inside and outside of caves, such as *Alluaudellina cavernicola* (Shelford, 1910) (ROTH 1988; ROTH & MCGAVIN 1994).

Biological knowledge of Nocticolidae is limited because the group is rare. In addition, the systematic position of Nocticolidae within Blattodea has been a topic of discussion for a long time. When BOLÍVAR (1892) described *Nocticola*, the cockroaches were only represented by Blattidae, and *Nocticola* was affiliated with the family. Although the placement of *Nocticola* in Blattidae was almost untouched for half a century (e.g., CHOPARD 1932; BEY-BIENKO 1950; REHN 1951), later PRINCIS (1966) listed the nocticolids as the family Nocticolidae in Blattoidea. ROTH (1988) discussed the affiliation of Nocticolidae based on the structures of the oothecae and genital organs and placed them between the Corydiidae (= Polyphagidae) and Blattellidae *sensu* MCKITTRICK (1964).

The cockroach group Nocticolidae is little known, and it has remained absent from molecular phylogenetic studies for a long time. LO et al. (2007) first performed a molecular phylogenetic examination of the genus *Nocticola*. They attempted to screen Dictyoptera, including three *Nocticola* species, with particular reference to the presence of the endosymbiont *Blattabacterium cuenoti* (Mercier, 1906), which resides in specialized cells, called bacteriocytes, of the fat body tissue of cockroaches. They revealed that Nocticolidae is a cockroach group that has not been infected by the endosymbiont and concluded that the family is a unique cockroach lineage. INWARD et al. (2007) conducted a large-scale molecular phylogenetic analysis and concluded that Nocticolidae and Corydiidae (= Polyphagidae) are sister groups, and they coined the name “Polyphagoidea” for the assemblage of these two families. WARE et al. (2008), who used molecular and morphological datasets for analysis, suggested that Nocticolidae and Corydiidae (and Lamproblattidae) are the most basal subgroups of Blattodea. From their combined morphological and molecular analysis, DJERNÆS et al. (2015) asserted that Nocticolidae should be a monophyletic group placed as the sister group to Latindiinae and nested within Corydioidea. More recently, BOURGUIGNON et al. (2018) suggested a sister group relationship between Nocticolidae and Corydiidae based on the complete mitochondrial genomes of 119 cockroach species, but some of the groupings did not have consistently high support. EVANGELISTA et al. (2019) analyzed a dataset comprised of ~2,370 protein-coding nuclear single-copy genes for 66 species, including all major groups of Blattodea except for Anaplectidae, and strongly supported the monophyletic Corydioidea.

More biological knowledge about Nocticolidae is required to reconstruct the groundplan and evolution of Blattodea. Nocticolidae may also have a special significance in the context of paleontology. The affinity of the enigmatic fossil group Miomoptera, which existed from the late Carboniferous to the late Triassic (MARTINS-NETO & GALLEGO 1999; PROKOP et al. 2017), has been highly debated and has ranged from “Protorthoptera” or stem-Polyneoptera to stem-Holometabola, due to the lack of conclusive evidence to support either hypothesis (see GRIMALDI & ENGEL 2005). BÉTHOUX & STONE (2011) inspected the wing system of a miomopteran, *Palaeomantis*, and suggested that Miomoptera is probably closely related to Nocticolidae: they found several special characters of wings shared by *Palaeomantis* and nocticolids. This finding suggests that at least a part of the “miomopteran” assemblage belongs to Blattodea. Thus, developing a better understanding of Nocticolidae also provides insight into an unresolved issue in insect evolution, which is essential to inferring the higher phylogeny and evolution of insects.

We examined the reproductive biology, such as mating behavior and handling of ootheca, and the embryonic development of a nocticolid, *Nocticola* sp. indet., from Malaysia. Here, we compare the information we obtained with that of other groups and discuss the phylogenetic issues regarding Nocticolidae and Blattodea.

2. Materials and methods

Eighteen individuals of a nocticolid cockroach were collected from a tropical rain forest in Selangor in Peninsular Malaysia in April 2011. They were reared in plastic cases (14 cm in diameter and 6.5 cm in height) with a moistened soil bottom kept at 25°C. The cockroaches were fed on a feed mixture of grained food for goldfish (Hikari-Flake Goldfish; Kyorin, Hyogo, Japan), a grained chlorella tablet (Chlorella; Fancl, Yokohama, Japan), a grained beer yeast tablet (EBIOS; Asahi Food & Healthcare, Tokyo, Japan), and grained balanced food (CalorieMate; Otsuka Pharmaceutical, Tokyo, Japan) at a mixing ratio of 9 : 3 : 3 : 1, as described by FUJITA et al. (2011), which established the rearing method for a corydiid, *Eucorydia yasumatsui* Asahina, 1971. We established captive breeding and obtained larvae, females and brachypterous and macropterous males (Fig. 1A–D). Inspection of the specimens from the captive breeding revealed that the present nocticolid species is characterized by the following features: 1) compound eyes present in macropterous males, vestigial in brachypterous males and females (Figs. 1C, 3B); 2) ocelli present only in macropterous males; 3) venation well developed in macropterous males (Fig. 1C); 4) wings absent in females (Figs. 1D, 3A,B); 5) abdominal terga II to IV specialized in males, relating to the tergal glands (Fig. 2A–D); and 6) styli of male subgenital lobe absent (Fig. 1A,B). Based on these features, we may be allowed to designate the present species as *Nocticola* sp. Referring to the unique specialization of the terga relating to the tergal glands, the present species is probably new to science, and we will describe it in the near future. The nocticolid species analyzed as *Nocticola* sp. in EVANGELISTA et al. (2019) is the same species as that used in the present study.

Living specimens, oothecae, and eggs of *Nocticola* sp. were photographed using a stereomicroscope (MZ12, Leica, Heerbrugg, Switzerland) equipped with a digital camera (Olympus E-620, Tokyo, Japan). The images were stacked using software Helicon Focus (Helicon Soft Ltd., Kharkiv, Ukraine).

For the observation of the specialization of the terga with the tergal glands, the brachypterous and macropterous males were mounted using Heinz medium (10 g polyvinyl alcohol + 80 ml distilled water (DW) + 35 ml lactic acid + 10 ml glycerin + 25 ml phenol + 20 g chloral hydrate) and examined under a biological microscope (Optiphot-2, Nikon, Tokyo, Japan).

The eggs were taken out of deposited oothecae in physiological saline Ephrussi-Beadle’s solution (0.75% NaCl + 0.035% KCl + 0.021% CaCl₂) and then fixed with Bouin’s fixative (saturated picric acid aqueous solution: formalin: acetic acid = 15 : 5 : 1) for 24 h or with Karnovsky’s fixative [2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1 M HCl-sodium cacodylate buffer, pH 7.2 (SCB)] for 24 h. Due to their fragility, the eggs in early developmental stages are easily broken when

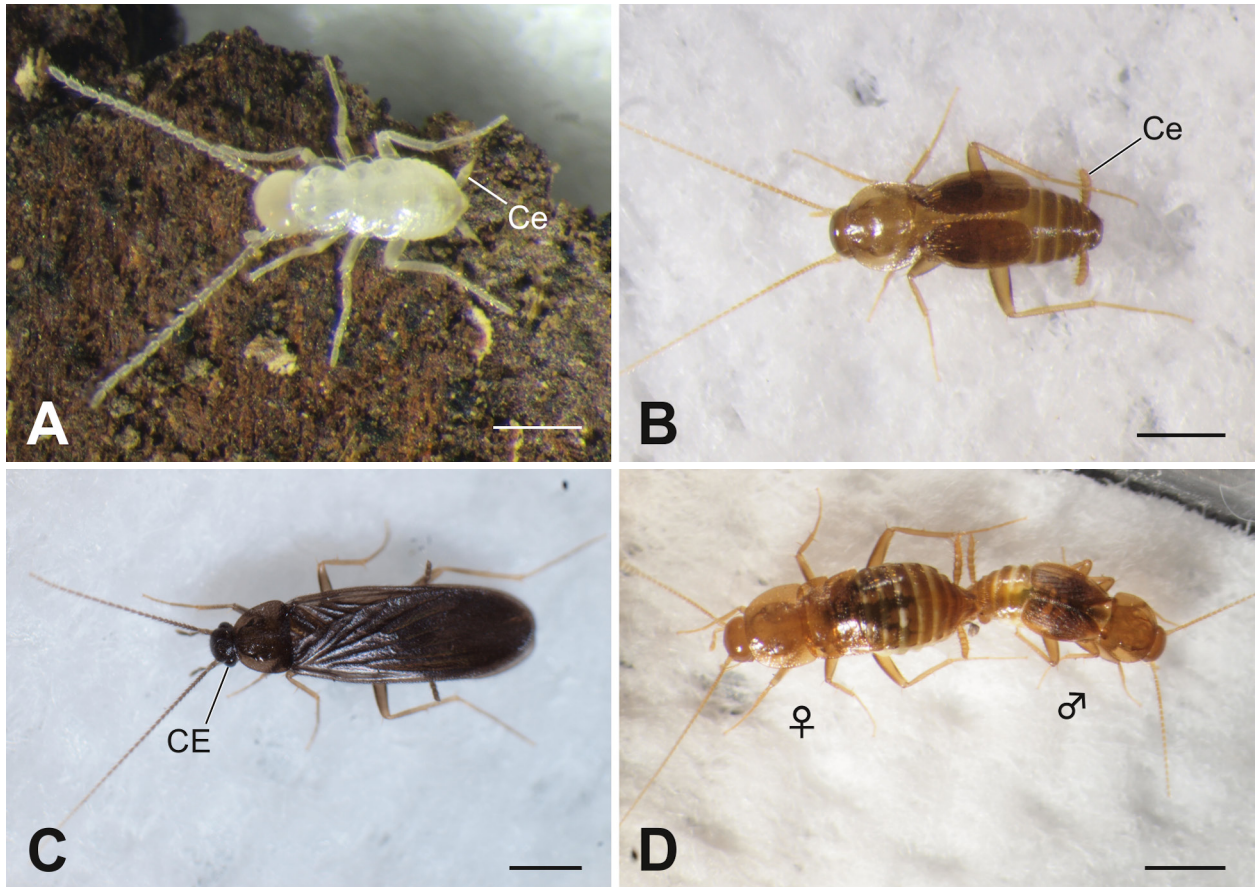


Fig. 1. The nocticolid species, *Nocticola* sp., examined in the present study. **A:** 1st instar larva. **B:** Brachypterous male. **C:** Macropterous male. **D:** Pair in copulation. — **Abbreviations:** CE – compound eye; Ce – cercus. — **Scale bars:** A – 0.5 mm; B–D – 1 mm.

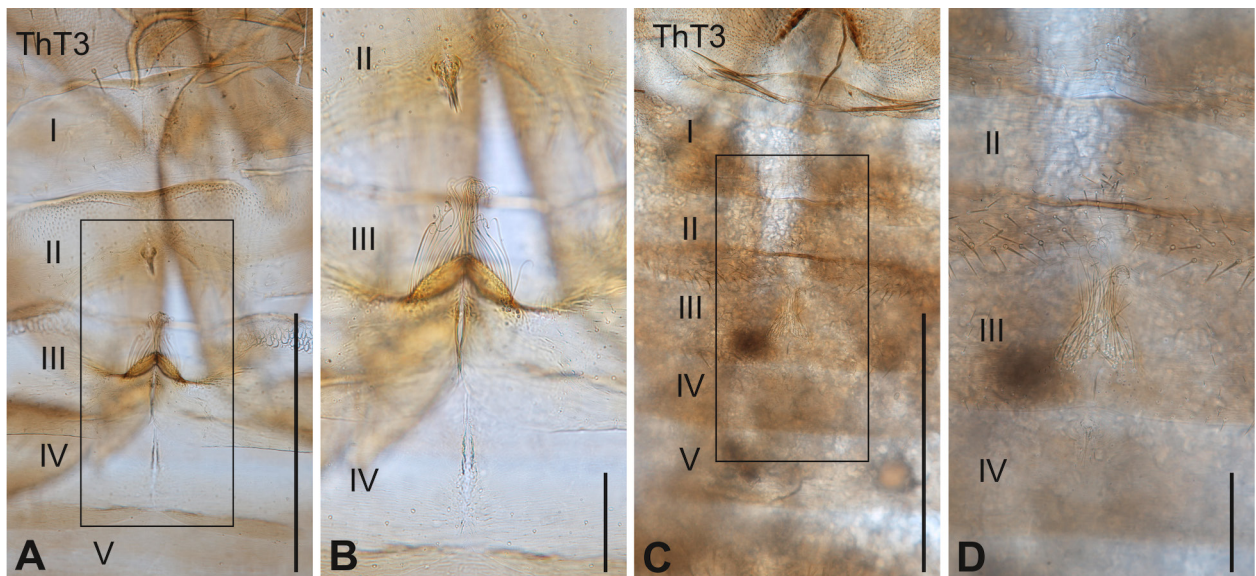


Fig. 2. Specialization of the abdominal terga, relating to tergal glands, in males of *Nocticola* sp. **A:** Brachypterous male. **B:** Enlargement of boxed area in A. **C:** Macropterous male. **D:** Enlargement of boxed area in C. — **Abbreviations:** ThT3 – methathoracic tergum; I–V – abdominal terga I to V. — **Scale bars:** A, C – 500 µm; B, D – 100 µm.

oothecae are dissected. In such cases, the oothecae that included young eggs were soaked in Bouin's or Karnovsky's fixative, and the eggs were punctured with a fine needle through the oothecal wall and subsequently fixed for 24 h. After the fixation, the eggs were carefully taken

out of the oothecae. The eggs fixed with Bouin's or with Karnovsky's fixative were then stored in 70% ethyl alcohol or in SCB.

Fixed eggs were stained with a DNA-specific fluorescent dye, DAPI (4',6-diamidino-2-phenylindole dihy-

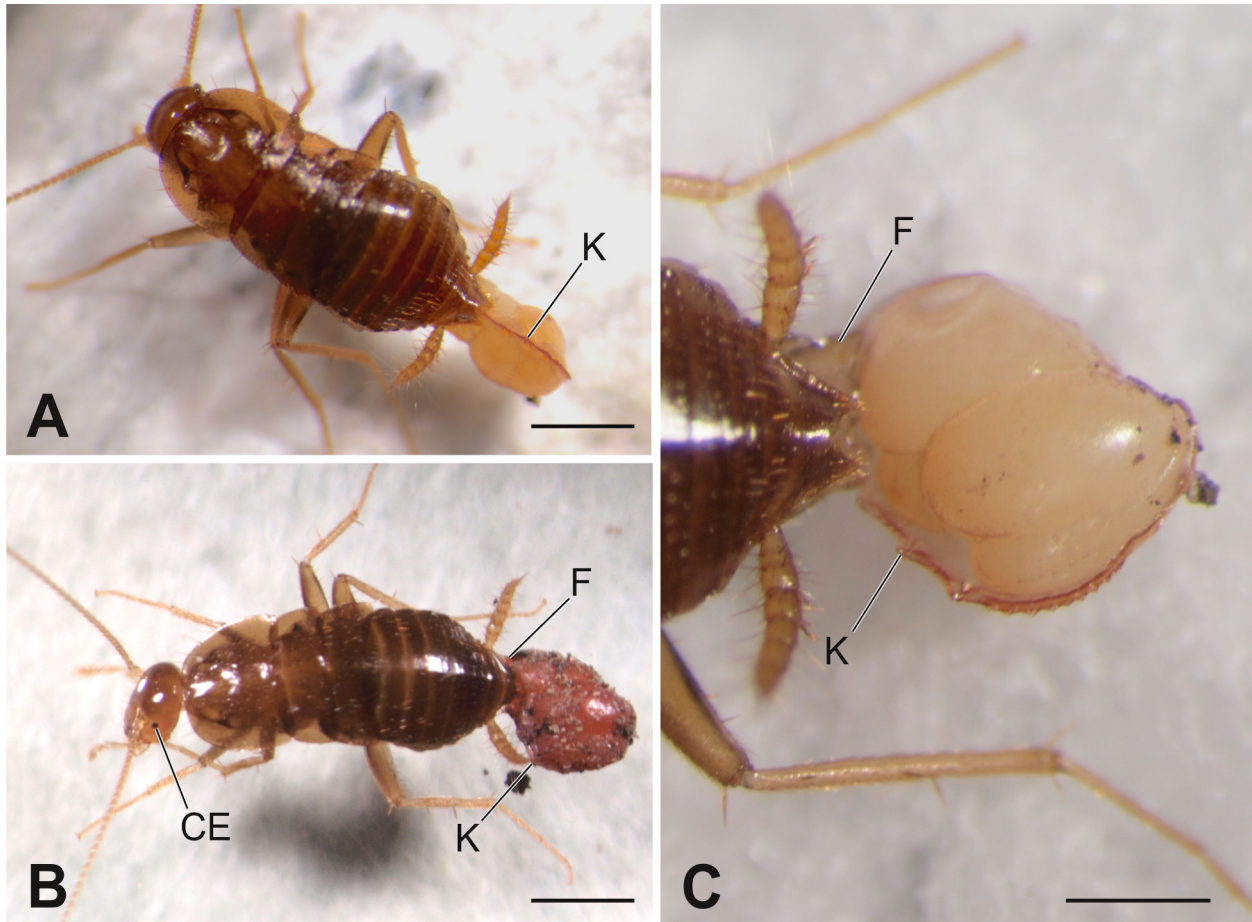


Fig. 3. Handling of ootheca in *Nocticola* sp. **A:** A female holding the ootheca with its keel upward. **B:** A female with the ootheca rotated by 90°. The flange of the ootheca is grasped by the caudal end of the female. **C:** Enlargement of the grasping of the rotated ootheca. — **Abbreviations:** CE – compound eye; F – flange; K – keel. — **Scale bars:** A, B – 1 mm; C – 0.5 mm.

drochloride, diluted to 10 µg/ml with DW) for 24 h to several days. Lipids in the eggs often hinder the staining solution from infiltrating. In such cases, it was more effective to soak the materials in acetone for several hours to remove the lipids. For this process, the samples were dehydrated using a graded ethyl alcohol series, kept in acetone for a period, then hydrated using the alcohol series in reverse, and finally transferred to the staining solution. Stained materials were observed with a fluorescence stereomicroscope (MZ FL III + FLUO COMBI, Leica, Heerbrugg, Switzerland) under UV-excitation at 360 nm.

For SEM observation of the egg, the fixed eggs were post-fixed with 1% OsO₄ for 2 h. They were dehydrated through a graded ethyl alcohol series and dried with a critical point dryer (Samdri-PVT-3D, tousimis, Rockville, USA). The dried specimens were mounted on the stub, coated with gold in an ion sputter (Ion Sputter JFC-1100, JEOL, Tokyo, Japan), and observed under an SEM at an accelerating voltage of 15 kV.

For the observation of micropyles, the chorions were cleaned using an ultrasonic cleaner, cut open, mounted with Heinz medium and then examined using a differential interference contrast microscope (DM6000B, Leica, Wetzlar, Germany).

3. Results

3.1. Mating behavior

The brachypterous males of the studied *Nocticola* sp. were often observed rapidly flapping their wings sideways around the female (see Electronic Supplement Movie S1) and then taking a position linearly opposite the female. The pair finally entered the mating in a “tail-to-tail” position (Fig. 1D). We did not observe the mounting of an individual on the back of its partner.

In the males, tergal specializations are found in abdominal terga II to IV; they are less pronounced in macropterous forms (Fig. 2C,D) than in brachypterous forms (Fig. 2A,B). They consist of a narrow, median, posteriorly directed process bearing a few stout and straight setae (on tergum II), which is ill-developed and difficult to discern in macropterous forms; a wide, median, anteriorly directed process bearing many long, apically curved setae, followed posteriorly by a median groove (on tergum III), and another median groove (on tergum IV), which is ill-developed and difficult to discern in macropterous forms. We did not observe macropterous males performing distinguished courtship, including flapping their wings and copulating.

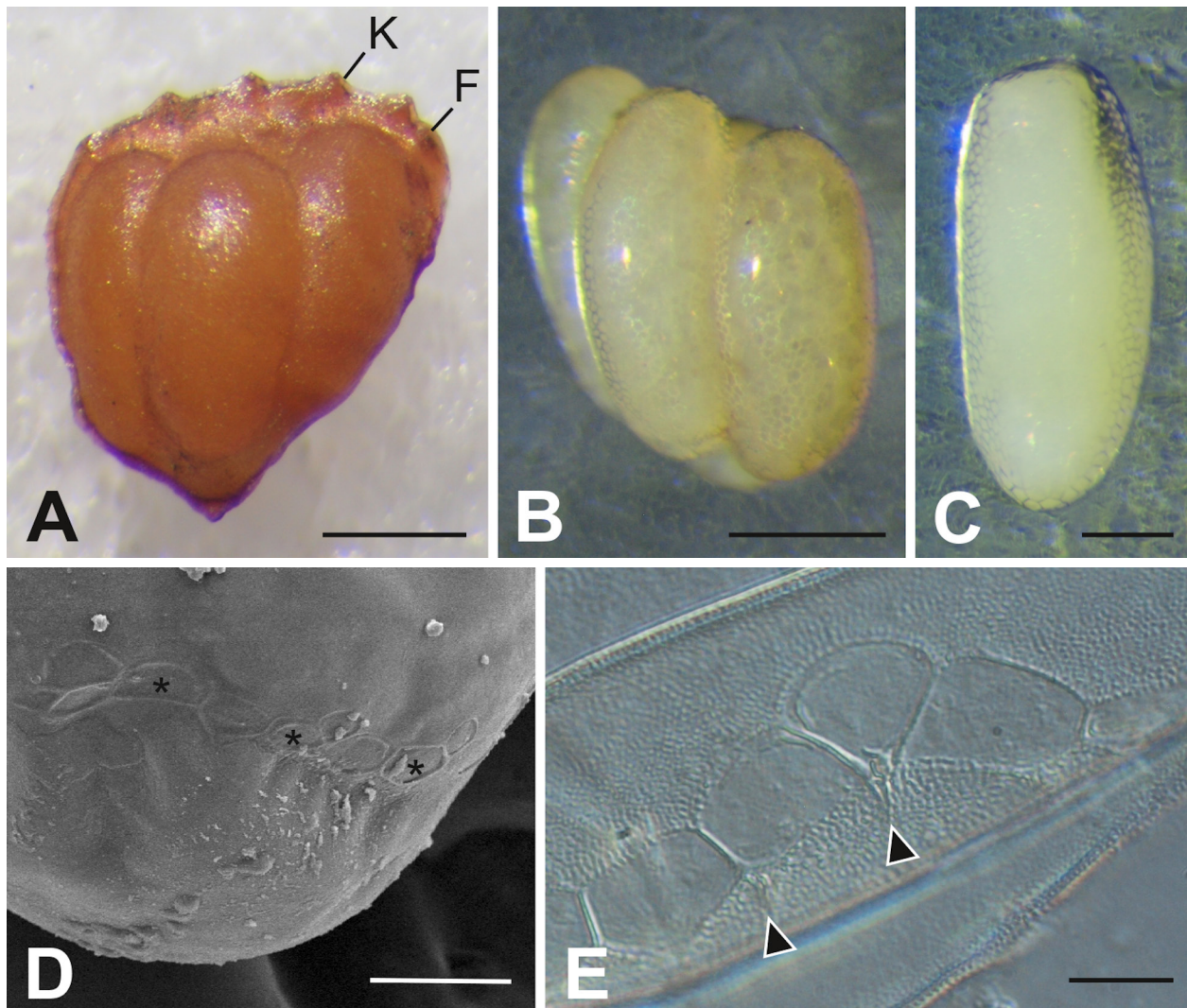


Fig. 4. Ootheca and eggs of *Nocticola* sp. **A:** Ootheca, lateral view, anterior to the left, dorsal to the top. **B:** Eggs removed from the ootheca, forming a mass, lateral view, anterior to the right, dorsal to the top. Four eggs are arranged with their ventral side facing each other. **C:** Egg, ventral view, anterior to the top. **D:** SEM of the posterior ventral side of an egg, anterior to the top. Asterisks show the polygon pattern on the posterior ventral side of the egg. **E:** Differential interference contrast microscopy of the connected-polygon pattern, anterior to the top. Arrowheads show micropyles. — **Abbreviations:** F – flange; K – keel. — **Scale bars:** A, B – 500 μ m; C – 200 μ m; D, E – 10 μ m.

3.2. Handling of ootheca

After copulation, the female of *Nocticola* sp. produces an ootheca and holds the ootheca with its keel upwards, grasping its flange with her caudal end (Fig. 3A, cf. Fig. 4A). Several hours later, the female rotates the ootheca clockwise by 90° viewed from the female's side, so that it takes a horizontal position (Fig. 3B,C). After rotation, the female carries the ootheca, holding it by its flange for a few days and maintaining this horizontal position, and then the ootheca is deposited on the ground. The ootheca is ever exposed and never hidden within the mother's vestibulum.

3.3. Ootheca and egg

The ootheca of *Nocticola* sp. (Fig. 4A) is approximately 1.2 mm long, 1.5 mm high, and 1.0 mm thick. In a newly

produced ootheca, eggs are visible through the translucent oothecal wall (Fig. 3C). Over time, the oothecal wall gradually becomes dark reddish (Figs. 3B, 4A). The ootheca bears a tiny, denticulated keel along its dorsal median line. The flange, which is grasped with the female's caudal end while she carries the ootheca, is ill-developed. The ootheca contains four eggs, which are arranged in two rows: the two eggs of one side are slipped by 1/2 egg's width against those of the other side (Figs. 3C, 4A,B). The dorsoventral axis of the ootheca corresponds to the anteroposterior axis of the eggs contained in it, and the dorsal side of an egg faces the oothecal wall.

The eggs are ellipsoidal, ca. 1.0 mm long and 0.4 mm wide, and whitish yellow (Fig. 4C). The egg's dorsal side is convex, and its ventral side, on which the embryo forms, is slightly concave (Figs. 4B, 6). A connected-polygon pattern is found at the ventral side of the egg near the egg's posterior pole (asterisks in Fig. 4D). About a dozen micropyles, each with a funnel-shaped entrance,

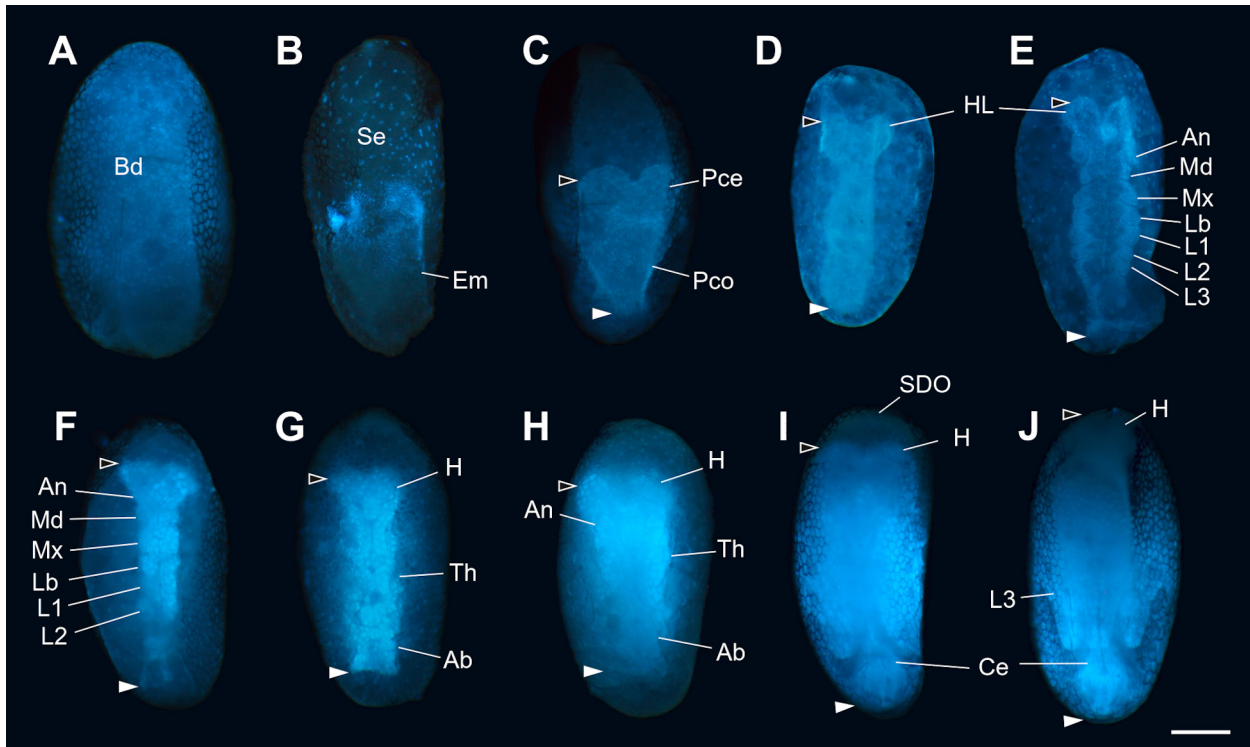


Fig. 5. Embryonic development of *Nocticola* sp., fluorescence microscopy (DAPI staining, UV-excitation), ventral views, anterior to the top, successive stages **A** to **J**. Black and white arrowheads indicate the anterior and posterior ends of the embryos, respectively. — **Abbreviations:** Ab – abdomen; An – antenna; Bd – blastoderm; Ce – cercus; Em – embryo; H – head; HL – head lobe; Lb – labium; L1–3 – prothoracic, mesothoracic, and metathoracic legs; Md – mandible; Mx – maxilla; Pce – protocephalon; Pco – protocorm; SDO – secondary dorsal organ; Se – serosa; Th – thorax. — **Scale bar:** 200 μ m.

are found on the posterior ventral side of each egg, and each micropyle is situated near the posterior angle of each polygon (Fig. 4E).

The eggs of cockroaches are known to contain an aggregation of symbiotic bacteria, known as a “mycetome” (see FUJITA & MACHIDA 2017). However, we found no signs of mycetomes in the eggs examined (see 4.2.1).

3.4. Embryonic development

The incubation period was 47.4 ± 0.5 days ($n = 7$) at 25°C . The cleavage is of the superficial type. The cleavage nuclei at the egg periphery divide further, and the cellularized blastoderm completes (Figs. 5A, 6A). The blastoderm soon differentiates into embryonic and extraembryonic areas. The embryonic area concentrates medially and posteriorly, and an oblong embryo forms in the posterior half of the ventral side of the egg (Figs. 5B, 6B). The extraembryonic area becomes the serosa (Figs. 5B, 6B). The embryo, in which the protocephalon and protocorm differentiate, starts to elongate along the ventral surface of the egg (Figs. 5C, 6C), and the embryo’s anterior end nearly reaches the anterior pole of the egg (Figs. 5D, 6D). The embryo undergoes segmentation from anterior to posterior, and the appendages differentiate in the differentiated segments (Figs. 5E,F, 6E,F). The embryo progressively grew with its caudal end bent anteriorly (Figs. 5G,H, 6G,H). The amnioserosal fold, which

was formed in the earlier stages (anatrepsis) as shown by Figs. 5D & 6D, is ruptured and withdrawn (katatrepsis). Then, the serosa anterodorsally concentrated to the anterior pole of the egg to form the secondary dorsal organ (Figs. 5H,I, 6H,I). Definitive dorsal closure proceeds, and the embryo grows further and acquires its definitive configuration (Figs. 5I,J, 6I,J).

The embryonic development of *Nocticola* sp. proceeds, with the embryo maintaining the original orientation in the egg and its ventral side facing the egg’s ventral side (Figs. 5, 6).

4. Discussion

4.1. Reproductive biology

4.1.1. Mating behavior and tergal glands

Cockroach mating behavior is often accompanied by elaborate courtship, specific to their group (see BELL et al. 2007), and their mating behavior has attracted considerable interest from researchers (ROTH & WILLIS 1954; ROTH & DATEO 1966; ROTH & BARTH 1967; ROTH 1969; KITAMURA 1974; SIMON & BARTH 1977). SRENG (1993) classified the mating behavior of cockroaches into three types: in type A, the male raises his wings, the female mounts the

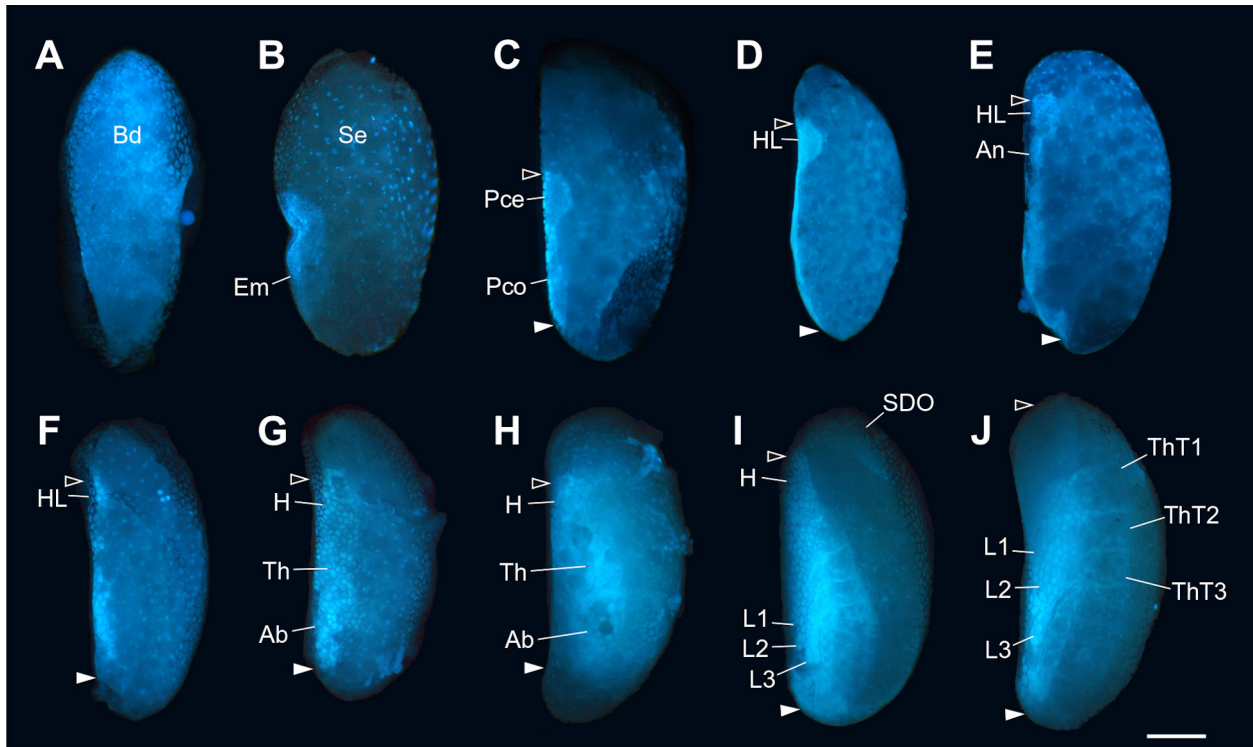


Fig. 6. Embryonic development of *Nocticola* sp., fluorescence microscopy (DAPI staining, UV-excitation), lateral views, anterior to the top, successive stages **A** to **J**. Black and white arrowheads show the anterior and posterior ends of embryos, respectively. — **Abbreviations:** Ab – abdomen; An – antenna; Bd – blastoderm; Em – embryo; H – head; HL – head lobe; L1–3 – prothoracic, mesothoracic, and metathoracic legs; Pce – protocephalon; Pco – protocorm; SDO – secondary dorsal organ; Se – serosa; Th – thorax; ThT1–3 – prothoracic, mesothoracic, and metathoracic terga. — **Scale bar:** 200 μ m.

male, the male engages the genitalia, and the pair enters the mating of the end-to-end position; in type B, the male mounts the female, engages the genitalia, and the pair enters the mating of the end-to-end position; and in type C, no mounting is performed, and the pair directly enters the mating in the end-to-end position. He suggested that type A, which is most widespread in cockroaches, is ancestral, and that the quite unusual types B and C are the derived. SRENG (1993) supposed that type B is an intermediate state between types A and C, but DJERNÆS et al. (2020) demonstrated that both types B and C appeared independently of each other from type A in several blattodean lineages.

Our study is the first to observe the mating of Nocticolidae. We did not observe the mounting of one individual on the back of its partner in a precopulatory sequence of *Nocticola* sp. Therefore, the mating behavior of Nocticolidae could be categorized as type C, whereas VIDLIČKA et al. (2017) presumed that the nocticolid *Helmablatta louisrothi* Vidlička, Vršanský et Deharveng, 2017 performs type A mating behavior based on its highly specialized abdominal terga. Notably, the mating behavior of type C may be quite widespread in Corydiidae [e.g., *Therea petiveriana* (Linnaeus, 1758) (LIVINGSTONE & RAMANI 1978); *Eucorydia yasumatsui* (FUJITA & MACHIDA 2014)], which are likely the closest relatives of Nocticolidae (see Introduction), though with the exception of at least *Ergaula capucina* (Brunner von Wattenwyl, 1893) (FRITZSCHE 1996), which performs type A mating behavior (see DJERNÆS et al. 2020).

The (brachypterous) males of *Nocticola* sp. were often observed rapidly flapping their wings sideways around a female. Such wing-flapping behavior, which has not been reported for other cockroaches, may facilitate the diffusion of pheromone from the tergal glands.

Tergal specializations associated with tergal glands are not known in the majority of Nocticolidae, but occur in some: on the metanotum in *Metanocticola christmasensis* Roth, 1999 (ROTH 1999), on abdominal terga II to IV in *Spelaeoblatta thamfaranga*, *S. myugei*, and *S. thailandica* Vidlička, Vršanský & Shcherbakov, 2003 (ROTH & MCGAVIN 1994; VIDLIČKA et al. 2003), on abdominal terga III to V in *Helmablatta louisrothi* (VIDLIČKA et al., 2017), on abdominal tergum IV in *Nocticola australiensis* Roth, 1988 and *N. uenoi* Asahina, 1974 (ASAHINA 1974; ROTH 1988), on abdominal terga III and IV in *Nocticola (Paraloboptera) rohini* (Fernando, 1957) (FERNANDO 1957), and on abdominal terga II to IV in *Nocticola currani* Trotter, McRae, Main & Finston, 2017 (TROTTER et al. 2017) and in *Nocticola* sp. studied herein.

Combined morphology and molecular analysis (DJERNÆS et al. 2015) and molecular phylogenetic analysis (LEGENDRE et al. 2015; WANG et al. 2017) suggested the sister group relationship between Nocticolidae and Latindiinae of Corydiidae. Notably, Latindiinae is the only group in the corydiid lineage whose males possess tergal specializations associated with the tergal glands: the three latindiine genera *Brachylatindia*, *Compsodes*, and *Melestra* have a specialized tergum IV (HEBARD

1921; PRINCIS 1952; ROTH 1969; QIU et al. 2019). However, the sharing of tergal glands or a specialized tergum by part of the nocticolid and latindiine lineages does not mean that Nocticolidae and Latindiinae are related. The tergal glands in blattodean males, which are closely linked with the mating behavior that is characteristic of Blattodea, are found in almost all blattodean families and likely groundplan structures of Blattodea. Therefore, the sharing of tergal specializations and glands by nocticolids and latindiines is likely a symplesiomorphy. The lack of such structures, which is sporadically found in several lineages among Blattodea, including Corydiidae, Blaberidae, Nocticolidae (ROTH 1969, 1988), and Isoptera except for Mastotermitidae (BORDEREAU & PASTEELS 2011) is likely derived.

In *Nocticola* sp. the macropterous males with ill-developed tergal specializations have well-developed compound eyes, whereas the brachypterous ones with well-developed tergal specializations have vestigial eyes. Therefore, macropterous males have sufficient locomotive and visual capability, but they are deficient in the efficient releasing of pheromone. However, brachypterous males have well-developed tergal glands, ensuring the efficient release of pheromone, but they lack eye sight and flight. The differences in locomotion, sight, and the function of releasing pheromone in brachypterous and macropterous forms could be a “trade-off.”

4.1.2. Handling of ootheca

Cockroaches produce oothecae, which each encapsulate one brood of eggs. BELL et al. (2007) classified the reproductive mode, including the handling of ootheca, into five types (see also table 1 of DJERNÆS et al. 2020): 1) oviparity A, in which the ootheca is dropped well before hatching and is carried externally for several days before deposition, is found in Corydiidae, Blattidae, Lamproblattidae, Tryonicidae, Cryptocercidae, Mastotermitidae, and majority of “Ectobiidae” s.l.; 2) oviparity B, in which the ootheca is carried externally until hatching, is found in Anaplectidae and a part of “Ectobiidae” s.l.; 3) ovoviviparity A, in which the ootheca is carried internally until hatching, without significant provision of nutrients from the mother, is found in the majority of Blaberidae; 4) ovoviviparity B, in which there are no oothecae, eggs are carried internally until hatching, without significant provision of nutrients from the mother, is found in part of Blaberidae; 5) viviparity, in which the eggs/ootheca are carried internally until hatching, with the eggs nourished by the mother, is found in part of Blaberidae.

In *Nocticola* sp., the ootheca is extruded in the keel-up position, soon rotated by 90°, carried for a few days by the mother, and finally deposited on the ground. This can be categorized as oviparity A, as also reported for *Nocticola australiensis* (personal communication to M. Djernæs, shown in Tables 1 and S5 of DJERNÆS et al. 2020). Corydiidae also show oviparity A (e.g., MCKITTRICK 1964; ROTH 1967; FUJITA & MACHIDA 2014). Furthermore Nocticolidae and Corydiidae share a unique

feature: the mother carries the ootheca holding its flange with her caudal end (MCKITTRICK 1964; ROTH 1967; FUJITA & MACHIDA 2014); this could suggest a close relationship between Nocticolidae and Corydiidae (as in, e.g., DJERNÆS et al. 2015; LEGENDRE et al. 2015; WANG et al. 2017; BOURGUIGNON et al. 2018; EVANGELISTA et al. 2019). As found for Nocticolidae, the ootheca is also rotated by 90° when it is carried by some corydiids, such as *Arenivaga* (*Arenivaga*) spp. (ROTH 1967), *Therea petiveriana* (ROTH 1967), and *Eucorydia yasumatsui* (FUJITA & MACHIDA 2014), suggesting a relationship between Nocticolidae and these corydiids.

4.2. Egg and embryonic development

4.2.1. Ootheca and egg

The ootheca of *Nocticola* sp. contains four eggs, is very short, and assumes a pear shape – like those of the nocticolids *Alluaudellina cavernicola* (CHOPARD 1932), *Nocticola* (*Paraloboptera*) *rohini* (FERNANDO 1957), and *Nocticola australiensis* (personal communication to M. Djernæs, shown in tables 1 and S5 of DJERNÆS et al. 2020).

The eggs of *Nocticola* sp. are dorsally convex and ventrally a little concave, similar to those of other cockroaches. In *Nocticola* sp., the micropyles, which are small pores for the entrance of sperm, are grouped in the posterior region of the ventral side of the egg. FUJITA & MACHIDA (2017) and FUKUI et al. (2018) suggested that the grouped micropyles on the ventral side of the egg are a groundplan feature of Dictyoptera, and that this is likely an apomorphic groundplan feature of this lineage, since such a distribution of micropyles is unique and has not been reported in other insects.

There are several patterns in the distribution of micropyles in cockroaches over the ventral side of the egg: they are located in the anterior region in *Blattella germanica* (Linnaeus, 1767) (Blattellidae s.s.) (FUJITA 2016), in the middle region in *Periplaneta fuliginosa* Serville, 1838 (Blattidae) and *Cryptocercus punctulatus* Scudder, 1862 (Cryptocercidae) (Thipaksorn & Machida unpublished), and in the posterior region in *Eucorydia yasumatsui* (Corydiidae) (FUJITA & MACHIDA 2017). *Nocticola* sp. and *E. yasumatsui* share the grouped micropyles in the posterior region, and this could support a close affinity between Nocticolidae and Corydiidae.

Cockroaches harbor symbiotic bacteria (*Blattabacterium*) in their fat bodies. The bacteria are transmitted vertically to offspring through transovarial transmission, and inside the egg, they form a mass called the “mycetome” (strictly the “bacteriome”) (e.g., GIER 1936; SABREE et al. 2009; FUJITA & MACHIDA 2017). After staining the eggs with a nucleic acid-specific dye, such as DAPI, the changes and migration of mycetomes with the progression of development can be traced from the beginning to around the katatrepsis stage, where the mycetomes break down and symbionts lodge in the mesoderm cells destined to

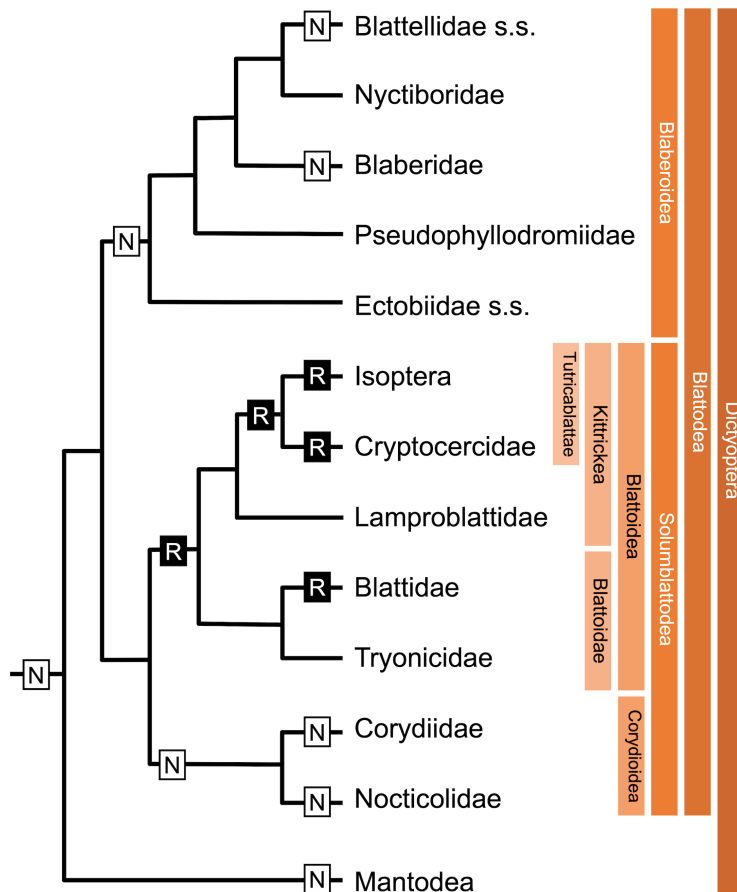


Fig. 7. Mapping of inferred blastokinesis types in Dictyoptera on a phylogenetic tree (simplified) proposed by EVANGELISTA et al. (2019); the relationships in Blaberoidea are re-organized according to DJERNÆS et al. (2020) “R” in black box and “N” in white box indicate the “reversion type” and “non-reversion type,” respectively. See text.

form the fat body. *Eucorydia yasumatsui* provides a good example of this process. The fig. 7a–h of FUJITA & MACHIDA (2017) explicitly demonstrates the behavior of mycetomes with progressive development in *E. yasumatsui*, in which the mycetomes are shown as the anterior mycetome (Amy), posterior mycetome (PMY), or mycetome (My). In the developmental stages in *Nocticola* sp. equivalent to fig. 7a–h in *E. yasumatsui* (Fig. 6A–H) we did not find any structures that we could recognize as mycetomes.

Screening dictyopterans, including three nocticolid species, for the presence/absence of *Blattabacterium*, LO et al. (2007) suggested that Nocticolidae is the only cockroach group uninfected by the endosymbionts. Our study is consistent with the findings of LO et al. (2007). The mycetomes were found in all investigated Blattodea (FUJITA & MACHIDA 2017), except for Nocticolidae (herein) and Isoptera under exclusion of their basalmost lineage Mastotermitidae (SACCHI et al. 1998). They are commonly regarded as one of the groundplan apomorphies of Blattodea (FUJITA & MACHIDA 2017). The loss of mycetomes in Nocticolidae and in Isoptera excluding Mastotermitidae has occurred independently (LO et al. 2007; FUJITA & MACHIDA 2017).

4.2.2. Blastokinesis

Two types of blastokinesis have been identified in Dictyoptera, i.e., the “reversion type” and the “non-reversion type” (for details, see FUJITA & MACHIDA 2017; FUKUI

et al. 2018). In the “reversion type,” which is known in the “blattarian” Blattidae and Cryptocercidae and in Isoptera (e.g., HEYMONS 1895; KNOWER 1900; STRIEBEL 1960; LENOIR-ROUSSEAU & LENDER 1970), the embryo, which formed in the ventral side of the egg, first elongates with its caudal end ahead beyond the posterior pole of the egg. As a result, the embryo takes its position in the dorsal side of the egg with its anteroposterior axis reversed, and undergoes development for a while. Then, the grown embryo returns back to the ventral side of the egg with its cephalic end ahead beyond the posterior pole of the egg, and the embryo recovers its original orientation (FUJITA & MACHIDA 2017: fig. 18a). In the “non-reversion type,” which has been identified in Blattellidae s.l. (of “Ectobiidae” s.l.), Blaberidae, and Corydiidae, and in the Mantodea, the sister group of Blattodea (e.g., WHEELER 1889; HAGAN 1917; BULLIÈRE 1969; TANAKA 1976; FUJITA & MACHIDA 2017; FUKUI et al. 2018), the embryo undergoes development with its ventral side facing the egg’s ventral side and without changing its orientation (FUJITA & MACHIDA 2017: fig. 18b). We previously attempted to reconstruct the phylogeny of Dictyoptera according to the type of blastokinesis. However, we failed to develop a conclusive determination, as we have no way of knowing whether the ancestor of Dictyoptera possessed the “reversion” or “non-reversion” type of blastokinesis (FUJITA & MACHIDA 2017).

The embryo of *Nocticola* sp. develops with its ventral side facing the egg’s ventral side and without changing

its anteroposterior axis throughout development. This represents the “non-reversion type.” The phylogenomic study by EVANGELISTA et al. (2019) proposed a robust phylogeny of Dictyoptera based on a dataset of ~2,370 protein-coding nuclear single-copy genes for 45 blattodean species, including all major groups except for Anaplectidae and 21 outgroup representatives. Fig. 7 is the simplified tree of EVANGELISTA et al. (2019; but relationships in Blaberoidea re-organized according to DJERNÆS et al. 2020), on which the inferred blastokinesis types for each lineage, i.e., the “reversion type” (R) and “non-reversion type” (N), are mapped. The type of blastokinesis of Dictyoptera is conformably understood following EVANGELISTA et al. (2019): 1) Corydioidea with N, in which Nocticolidae and Corydiidae share N; 2) Due to its high diversity much more information on the blastokinesis type needs to be accumulated for Blaberoidea, but based on currently available data Blaberoidea with N, in which Blattellidae s.s. and Blaberidae share N; 3) Tutricablattae with R, in which Isoptera and Cryptocercidae share R; 4) Blattoidea with R, in which Tutricablattae and Blattidae share R; and 5) the ancestral blastokinesis type in Dictyoptera would have been N in light of parsimony. Considering that the blastokinesis type is predominantly R in “Hemimetabola” (see FUJITA & MACHIDA 2017), the non-reversion type of blastokinesis (N) could be regarded as an autapomorphy of Dictyoptera. The manner of ootheca handling, the arrangement of micropyles, and the mating behavior discussed above support Corydioidea being monophyletic (see 4.1.1., 4.1.2. and 4.2.1.): the same applies to the blastokinesis type.

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Authors' contributions

M.F. and R.M. designed the study. All authors contributed to collecting materials. M.F. conducted the observations and drafted the manuscript together with R.M. All authors discussed the results and approved the final version of the manuscript.

Electronic Supplement File

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File 1: FujitaEtAl-Nocticola-ASP2020-ElectronicSupplement-1.mov — **Movie S1.** Pre-copulatory behavior of brachypterous male, filmed with a smartphone (LYNX 3D SH-03) camera. The male rapidly flaps his wings sideways around a female. We failed to confirm whether this pair, the female of which looks teneral, could establish a population or not.

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