A rapidly progressing, deadly disease of *Actias selene* (Indian moon moth) larvae associated with a mixed bacterial and baculoviral infection

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The outbreak of an infectious disease in captive-bred Lepidoptera can cause death of all the caterpillars within days. A mixed baculoviral–bacterial infection observed among *Actias selene* (Hübner 1807), the Indian moon moth (Insecta: Lepidoptera: Saturniidae), larvae was characterized and followed by a photographic documentation of the disease progression. The etiological agents were determined using mass spectrometry and polymerase chain reaction (PCR). It appeared that the disease was caused by a mixed infection of larvae with a baculovirus and *Morganella morganii*. A molecular phylogenetic analysis of the virus and microbiological description of the pathogenic bacterium are presented.

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1. Introduction

Infectious diseases can lead to serious losses in commercially bred Lepidoptera, for example, in silkworm farms. Infectious diseases can be caused by viruses, bacteria, fungi, protists or nematodes. Viruses belonging to the Baculoviridae family are entomopathogens known as 'zombie' viruses because they manipulate the host's behaviour – they force their victim (Lepidopteran, Dipteran or Hymenopteran) (Bergold 1953; Rennie 1923; Cunningham and Entwistle 1981) larva to climb up their food plant, then cause the insect's body to liquefy and thus spread millions of viral particles onto foliage below, where they can be eaten by further larvae. Unlike many entomopathogenic bacteria, baculoviruses are incapable of infecting vertebrates and rarely infect species beyond the genus or family of the original host (Gröner 1986). Bacterial infections cause different signs and symptoms, which may include vomiting and diarrhoea, cessation of feeding and paralysis, body flaccidity or cuticle melanization, as an effect of the caterpillar's defence mechanism (Cerenius and Söderhäll 2004).

Actias selene, belonging to the Saturniidae family, is a species of silkmoth commonly reared in butterfly farms due to its impressive size (wingspan up to 182 mm) and colouration (Hampson 1892). First instar larvae are red with a black fragment on the dorsal side, which is not visible in second instar caterpillars. After their second moulting, the larvae become bright green with yellow tubercles (figure 1). Imagines have pale green or yellow wings with slightly curled tails on the hindwings (Hampson 1892). A rapidly spreading infectious disease has been observed in *A. selene* larvae bred in a butterfly farm and is described here.

2. Materials and methods

2.1 *Monitoring the course of disease*

Healthy *A. selene* larvae were reared in a butterfly farm in netted cages containing their food plant (*Crataegus*) placed in a jar of water. Any larvae with even the slightest colouration

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Figure 1. Different stages of development of *Actias selene* larvae. The two smallest larvae are first instar, the middle-sized larva is the second instar, and largest larva is the third instar.

abnormalities were immediately isolated from healthy caterpillars. Ten larvae with infection symptoms were placed in containers previously sanitized with an aqueous solution of 2% sodium hypochlorite and 16.5% sodium chloride. The disease progression was monitored and photographed every 24 h.

2.2 *Bacteria isolation and identification*

Cuticle surfaces of larvae (1) dead due to infection (two individuals) and (2) alive and without infection symptoms (1 individual) were imprinted and streaked on agar plates with Luria-Bertani growth medium (Sambrook *et al.* 1989) and incubated for 48 h in the same conditions as *A. selene* larvae were reared: 28°C during the day and ~20°C during the night. Dominant bacteria, i.e. potential pathogens, were selected for further studies. Optimal growth temperature was determined by streaking overnight liquid cultures on 2 × YT agar plates (Sambrook *et al.* 1989) and incubating at a range of temperatures from 30°C to 45°C. Antibiograms were performed using the disk diffusion method on 2 × YT agar plates. Morphology of the bacteria was determined by Gram staining. The microorganisms were identified via the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) method by Bruss Laboratories (Gdynia, Poland).

2.3 *Baculoviral DNA detection*

Baculoviral DNA was isolated from the body fluids mixed with excretions of three diseased *A. selene* caterpillars using QIAGEN'S QIAamp DNA Mini Kit following the 'Free viral DNA from fluids or suspensions' protocol. In order to determine whether baculoviral genes were present in the isolated DNA, polymerase chain reactions were carried out. Three sets of primers, originally constructed by Lange *et al.* (2004) and proven to detect lepidopteran-specific nuclear polyhedrosis viruses and granuloviruses, were used (table 1). Optimized reaction composition (per 50 µL) was as follows: 0.2 µM primers (each), 100 µM deoxynucleotides, 2 mM $MgCl_2$, 6% DMSO, Taq buffer with $(NH_4)_2SO_4$, 1 unit of Taq polymerase, 0.3 µL template DNA. The volume of the mixture was adjusted to 50 µL with sterile water.

Optimal annealing temperatures were determined by running preliminary PCR reactions in a range of temperatures. PCR products were visualized through agarose gel electrophoresis, DNA purification and sequencing was performed at Genomed, Warsaw, Poland.

2.4 *Sequence alignment and phylogenetic analysis*

The newly generated partial sequences of polyhedrin and late expression factor 9 genes (GenBank Accession numbers are given in table 2) were compared to the sequences available in GenBank database *http://www.ncbi.nlm.nih.gov/BLAST/* using BLASTN search (Altschul *et al.* 1990) in order to confirm their identity. The sequences were aligned with sequences of selected representatives of nucleopolyhedroviruses (NPVs) (according to Jehle *et al.* 2006) obtained from GenBank and *Peridroma morpontora* GV that was used as an outgroup in further analyses. Alignment was performed using Seaview software (Galtier *et al*. 1996; Gouy *et al.* 2010) employing the clustalw2 option and followed by

Table 1. Primer sequences and PCR conditions used for baculoviral DNA detection

Target gene	Primer name/sequence	PCR conditions
Polyhedrin/granulin	prPH-1/TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT prPH-2/CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT	Initial denaturation: 95ºC/3 min Denaturation: 95ºC/30 s Annealing: 50ºC/1 min Elongation: 70ºC/1 min (36 cycles) Final elongation: 72ºC/10 min Hold cycle: 4ºC/until analysis
Late expression factor 8	prL8-1/CAGGAAACAGCTATGACCCAYGGHGARATGAC prL8-2/CAGGAAACAGCTATGACCAYRTAS1GGRTCYTCS$_2$GC	Initial denaturation: 95ºC/4 min Denaturation: 95ºC/2 min Annealing: 38-48ºC/1 min Elongation: 72ºC/1 min (35 cycles) Final elongation: 72ºC/2 min Hold cycle: 4ºC/until analysis
Late expression factor 9	prL9-1/CAGGAAACAGCTATGACCAARAAYGGITAYGCB$_3$G prL9-2/TGTAAAACGACGGCCAGTTTG4TCDCCRTCRCARTC	Initial denaturation: 95ºC/4 min Denaturation: 95ºC/2 min Annealing: 45-54ºC/1 min Elongation: 72ºC/1 min (35 cycles) Final elongation: 72ºC/2 min Hold cycle: 4ºC/until analysis

manual optimization. Portions of the alignment with ambiguous positions that might not have been homologous were eliminated. The phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 2001) with neighbour-joining (NJ) and maximum parsimony (MP) as optimality criteria. Heuristic searches were performed with 1000 random sequence additions and TBR branch swapping. Gaps were treated as missing and the support for the branches was tested with the bootstrap method with 1000 pseudoreplicates.

Maximum likelihood (ML) analyses were performed with the fast likelihood software PhyML 3.0 (Guindon and Gascuel 2003; Guindon *et al.* 2005), starting with a BioNJ tree. The GTR+I+G model of evolution was selected based on Hierarchical Likelihood Ratio Tests and Akaike Information Criterion in Modeltest 3.5 (Posada and Crandall 1998) and used in analysis. Bootstrap analyses were performed with 1000 replicates.

The phylogenetic tree was drawn using TreeView (Page 1996). Bootstrap supports (in NJ, MP and ML) greater than or equal to 70% were considered as strongly supported and were indicated near the branches.

3. Results and discussion

3.1 *Infection outbreak*

The described infection occurred among *A. selene* larvae bred in a butterfly farm. It started from only several caterpillars out of a few dozens and spread rapidly, causing death in all cases of larvae with visible symptoms. Ten infected caterpillars were selected for detailed observations and studies.

3.2 *Course of disease*

Upon the occurrence of the first dark spots on the cuticle, the caterpillars fed and displayed normal mobility. Starting from day 2, cuticle melanization expanded (figure 2), bodies became less resilient and the larvae displayed digestive disturbances: vomiting and diarrhoea. Although the integument was severely darkened, it remained intact. The larvae stopped feeding, became less mobile, flaccid and eventually completely paralysed. Moreover, when punctured after death, body liquefaction was revealed. The vast majority of

Table 2. Viruses and GenBank Accession Numbers of their sequences of polyhedrin and late expression factor 9 (lef-9) genes used in phylogenetic analysis

Virus	Polyhedrin gene	Late expression factor 9 gene
ActiasseleneS1	*AY706680*	*AY706592*
Actias seleneS2	*AY706678*	*AY706590*
Agraulis sp.	*AY706682*	*AY706600*
Agrotis segetumA123	*AY706683*	*AY706600*
Amorbia cuneacapsaA8-3	*AY706685*	*AY706602*
Anagrapha falciferaA5-3	*AY706686*	*AY706604*
Antheraea pernyiS5	*AY706687*	*AY706605*
Apocheima cinerariusS7	*AY706688*	*AY706606*
Aporia crataegiM45-3	*AY519210*	*AY519212*
Autographa californicaA122	*AY706681*	*AY706593*
Autographa californicaS43	*AY706679*	*AY706591*
Bombyx moriM28-4	*AY519216*	*AY519218*
Bombyx moriS12	*DQ231339*	*DQ231341*
Bombyx moriS9	*DQ231336*	*DQ231338*
Coloradia pandoraM30-2	*AY519228*	*AY519230*
Coloradia pandoraM30-2	*AY706690*	*AY706610*
Dirphia peruvianusA3-1	*AY706691*	*AY706611*
Ectropis grisescensS22	*AY706692*	*AY706612*
Euproctis digrammaS24	*AY706693*	*AY706614*
Galleria mellonellaA11-3	*AY706696*	*AY706618*
Galleria mellonellaA16-3	*AY706697*	*AY706619*
Galleria mellonellaA3-6	*AY706698*	*AY706620*
Hemerocampa vetustaA24-5	*AY706699*	*AY706623*
Hyphantria cuneaS27	*AY706700*	*AY706625*
Junonia coeniaM30-5	*AY519234*	*AY519236*
Lymantria monachaA14-3	*AY706701*	*AY706627*
Lymantria monachaA19-3	*AY706702*	*AY706628*
Lymantria xylinaS31	*AY706703*	*AY706629*
Malacosoma americanumM39-4	*AY706704*	*AY706630*
Malacosoma neustria S32	*AY706708*	*AY706634*
Mamestra brassicaeA10-1	*AY706705*	*AY706631*
Mamestra brassicaeA3-5	*AY706706*	*AY706632*
Mamestra brassicaeS33	*AY706707*	*AY706633*
Nepytia phantasmariaA25-5	*AY706709*	*AY706636*
Peridormamorpontora	*AY706672*	*AY706638*
Peridroma margaritosaA25-4	*AY706710*	*AY706637*
Phryganidia californicaM36-3	*AY519249*	*AY519251*
Plusia acutaA14-5	*AY706712*	*AY706642*
Plutella maculipennisA15-2	*AY706713*	*AY706643*
Pterolocera amplicornisM35-2	*AY519255*	*AY519257*
Samia cynthiaS36	*AY706711*	*AY706639*
Spilosoma phasmaS3	*AY706684*	*AY706601*
Spodoptera littoralisA26-5	*AY706717*	*AY706650*
Spodoptera littoralisA9-1	*AY706718*	*AY706651*
Spodoptera lituraA17-3	*AY706714*	*AY706645*
Spodoptera lituraS37	*AY706715*	*AY706646*

Table 2 (continued)

Virus	Polyhedrin gene	Late expression factor 9 gene
Spodoptera terricolaA26-1	*AY706716*	*AY706647*
Thysanoplusia orichalceaA28-1	*AY706719*	*AY706652*
Tineola bisselliellaM50-4	*AY706720*	*AY706653*
Virus from this study	*KR057236*	*KR057235*

the observed infected *A. selene* caterpillars were in their last instar. In all of the studied cases, this acute disease led to the death of larvae within 4–6 days from the appearance of the first signs. A few individuals survived long enough to spin a cocoon and died before pupation. The observed signs and symptoms differed from those occurring as a cause of a typical baculoviral infection. Above all, the larvae did not display climbing behaviour, which is known to be baculovirus-induced (Hamblin and Tanaka 2013). Their bodies were liquefied after death but never dripping onto lower parts of the food plant (due to lack of mobility and paralysis). This suggested, along with the melanised, yet intact integument, that the observed disease was caused by bacteria.

3.3 *Isolation and characterization of bacteria*

Cuticle surface imprints revealed the presence of bacteria on infected caterpillars. A low number of bacterial colonies was also visible on the control plate, with an imprint of a larva without disease symptoms (figure 3). These bacteria could belong to the caterpillar's physiological flora. However, it must be noted that healthy larvae were initially reared in the same cage as caterpillars which later showed disease symptoms. Thus, although without visible symptoms, the larvae could be in an early stage of infection, and hence the presence of bacteria on the control plate. The potentially pathogenic bacterial colonies had a whitish, opalescent colouration and a circular form. Gram staining revealed Gram-negative coccobacilli. Growth temperature optimization indicated that the microorganism is a mesophile which can grow in temperatures up to 42°C. The disk diffusion method showed resistance to ampicillin, oxacillin, bacitracin, cefpodoxime and the antifungal agents nystatin and natamycyn. Tetracycline, kanamycin and cefoperazone caused growth inhibition.

The bacterial strain was identified using the MALDI TOF MS method with high credibility (identification index 2.606) as *Morganella morganii* sp. *sibonii*. This species has been previously reported as an insect pathogen (Nishiwaki *et al.* 2007). The fact that *M. morganii* was the most numerous microorganism grown from cuticle imprints and the presence of symptoms characteristic for bacterial infections in *A. selene* larvae indicates that this bacteria was the main etiological factor of the studied infection.

The liquefied entrails, as well as faeces of infected *A. selene* larvae were also streaked on agar plates. After incubation, whitish bacterial colonies, morphologically identical to those from cuticle surface imprints, were visible.

Figure 2. *Actias selene* larva's disease progression. Day 1 – first disease signs: individual dark spots on the cuticle; days 2 and 3 – extending melanization, vomiting and diarrhoea, paralysis; day 4 – almost complete melanization of the cuticle, death.

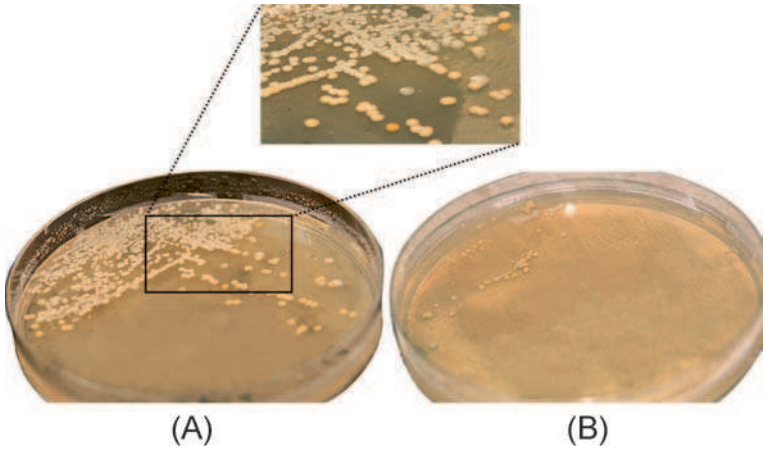

Figure 3. Cuticle surface imprints of *Actias selene* larvae (**A**) with disease symptoms and (**B**) without disease symptoms.

M. morganii may cause gastrointestinal disease in humans (O'Hara *et al.* 2000). Nevertheless, its presence has also been reported in healthy people (Müller 1986). Because the studied *A. selene* larvae were reared in a butterfly farm, it is possible that the infection originated from a person handling the caterpillars or their food plants. However, *M. morganii* are also ubiquitous environmental/fecal microorganisms and thus it remains to be determined how they appeared in *A. selene*.

The second most numerous bacterial strain from the initial *A. selene* cuticle imprints was also subjected to identification using the MALDI TOF MS method. It was classified as *Corynebacterium casei*; however, the value of the identification index (1.785) indicates a precise genus level and a probable species level identification. Thus, it will be further referred to as *Corynebacterium* sp. The presence of lipase-producing *Corynebacterium* has been previously reported in the intestines of silkworms, *Bombyx mori* (Feng *et al.* 2011) as physiological flora. Feng *et al.* (2011) suggested that the secreted lipases may provide a NPV resistance.

3.4 *Determining the presence of baculoviruses*

The larvae selected for examination displayed syndromes characteristic for a bacterial infection. However, it is worth noting that before they were placed in separate containers in

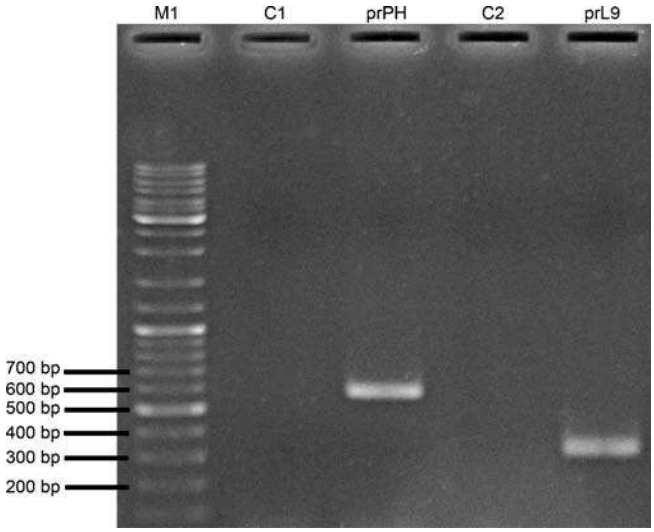

Figure 4. Detecting baculoviral DNA: agarose gel electrophoresis of PCR products obtained using polyhedrin/granulin(prPH) and late expression factor 9 (prL9) primers. Lane M1 – GeneRuler^{TM} DNA Ladder Mix Ready-to-Use, 100-10000 bp (selected bands marked); lane C1 – PCR with prPH primers without DNA from *A. selene* sample; lane prPH – PCR with prPH primers with DNA from *A. selene* sample; lane C2 – PCR with prL9 primers without DNA from *A. selene* sample; lane prL9 - PCR with prL9 primers with DNA from *A. selene* sample.

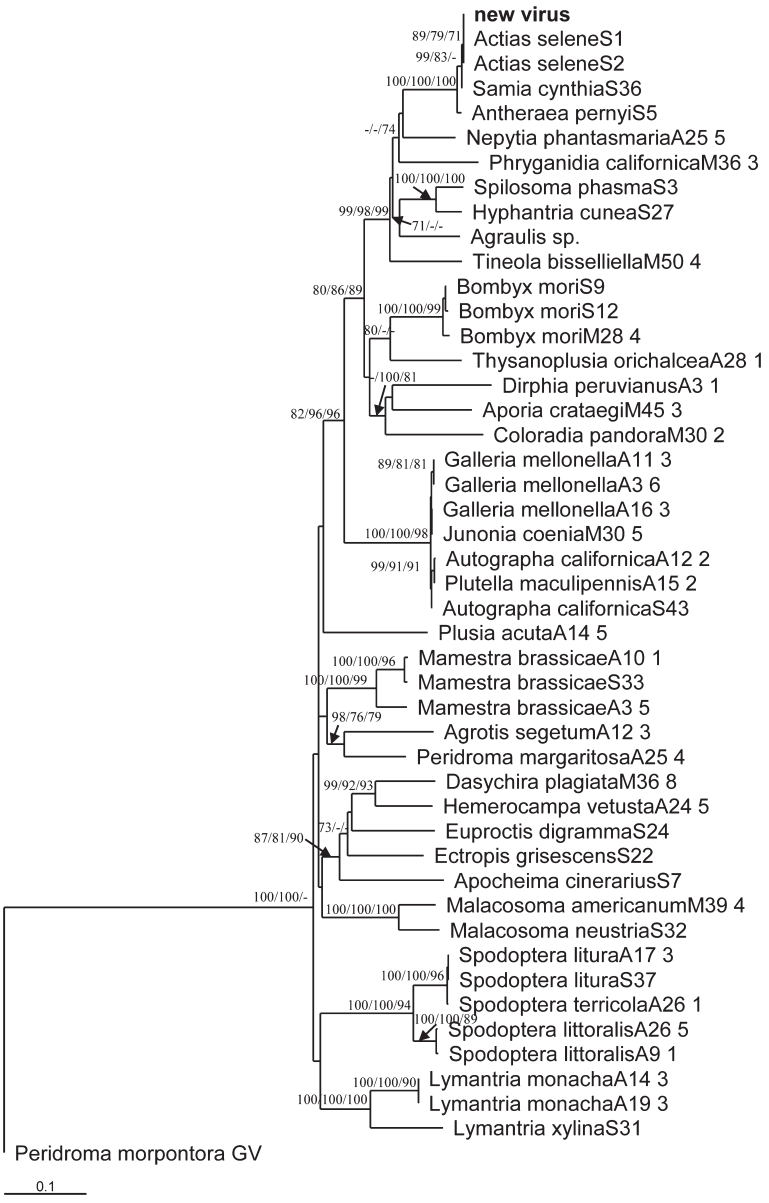

Figure 5. Neighbour-joining tree based on concatenated DNA sequences of the partial *polyhedrin* and *lef-9* genes of selected baculoviruses. Numbers at the nodes represent bootstrap values (1000 replicates) from neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods, respectively. Only values greater than or equal to 70% are presented. *Peridroma morpontora* GV was used as an outgroup. The virus identified in this study is indicated in bold.

order to monitor the infection's progression, they were reared in netted cages in a butterfly farm. Thus, the transmission of infectious diseases was possible. Other species of Lepidoptera kept at the butterfly farm displayed symptoms characteristic for baculovirus infections.

In order to determine whether baculoviruses were also involved in the observed disease, samples of body fluids mixed with excretions from larvae dead of the observed infection were tested for the presence of baculoviral genes. Electrophoresis (figure 4) of PCR products obtained using as a template DNA isolated from these samples revealed the presence of two highly conserved baculoviral genes, polyhedrin/granulin (*polh*) and late expression factor 9 (*lef9*), in two out of three studied samples. PCRs run with late expression factor 8 primers did not amplify any DNA in any of the studied cases.

DNA sequencing resulted in 544 bp reads generated by prPH primers and 298 bp reads generated by prL9 primers, both being longer than the gene sequences obtained by Lange *et al.* (2004). This might mean that the baculoviral genes present in the samples contained an insertion within the *polh* and *lef9* genes. The obtained sequences were compared with the nucleotide database of the National Center for Biotechnology Information (NCBI). The comparison revealed 100% identity of aligned reads obtained from the prPH primers with *A. selene* NPV *polh* gene (GenBank: AY706678.1). Aligned sequences obtained from the prL9 primers were 100% identical with *A. selene* NPV *lef9* gene (GenBank: AY706590.1).

Phylogenetic analysis of selected representatives of NPVs (figure 5) showed that the virus identified in samples analysed in this work forms a single, highly supported group with two other *Actias selene* NPVs (isolates S1 and S2), that are closely related to viruses reported from *Samia cynthia* (isolate S36) and *Antheraea pernyi* (isolate S5) hosts. Phylogenetic analysis and BLASTn search confirmed that the virus present in *Actias selene* larvae analysed in this work represents the same virus which has previously been reported from an *Actias selene* host by Jehle *et al.* (2006).

4. Conclusions

These results unquestionably indicate that both bacteria and baculoviruses were present in the infected *A. selene* larvae tissues. It cannot be clearly established which infected the larvae first. An initial baculoviral infection may have weakened the immune system of the larvae, allowing the bacteria to attack and eventually kill the host. However, as previously mentioned, bacteria belonging to the genus *Corynebacterium* have been proved to produce lipases that could develop resistance to baculoviral infections. Since they were present on the studied *A. selene*'s cuticle, an inverse situation might have occurred: *M. morganii* caused the initial infection, making the caterpillar's defence mechanisms less efficient, and enabling baculovirus infection. Regardless of the order, one may conclude that the studied disease originated from a mixed baculoviral–bacterial infection, whereas the action of *M. morganii* mainly induced larvae mortality.

These studies draw attention to the fact that common environmental bacteria may be a cause of serious infection outbreaks in insects, and caution must be taken when rearing and studying invertebrates, as entomopathogens may be disseminated by humans.

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