Natural enemies of insect production systems:

where are risks and how can we address the risks?

Jørgen Eilenberg

Department of Plant and Environmental Sciences
University of Copenhagen, Denmark

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Outline

- Types of natural enemies and their importance
- A survey of insect diseases in commercial production
- Challenges in closed, semi-open and open production facilities
- Diagnostic, methods and a case study: Zophobas and bacteria
- Probiotics
- Insect diseases, are they harmful to humans
- European collaboration

Diseases in insects produced for food and feed

J. Eilenberg[1*], J.M. Vlak[2], C. Nielsen-LeRoux[3], S. Cappellozza[4] and A.B. Jensen[1]

Journal of Insects as Food and Feed, 2015; 1(2): 87-102

Some of our 2015-2017 publications on this subject

Natural enemies in insect production systems

J. Ellenberg[1*], S.N. Gasque[1] and V.I.D. Ros[2]
[1]Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C., Denmark; [2]Laboratory of Virology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands; jei@plen.ku.dk

In Huis, A. (ed) 2017: Insects as Food and Feed from Production to Consumption. Wageningen University Press, 201-222.

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Prevention and Management of Diseases in Terrestrial Invertebrates

Jørgen Eilenberg and Annette Bruun Jensen

Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark

In Hajek, A.E. & Shapiro-Ilan, D. (eds) in press: Ecology of Invertebrate Diseases, John Wiley and Sons

Natural enemies of red palm weevil

Rhynchophorus ferrugineus

Big vertebrate predators; small arthropod predators; parasitoids; phoretic mites; nematodes; insect pathogenic fungi, virus, microsporidia, and bacteria.

Table 10.1. Characteristics of selected main taxa of natural enemies of insects. Generalists: infecting or predating on hosts from taxonomically unrelated taxa (for example different insect orders). Specialists: infecting or predating either just one host species or several taxonomically closely related host species.

Natural enemy taxa	Mode of action	Host specialization
Predators:		
vertebrates (birds, reptiles, mammals) or invertebrates (insects, spiders and mites)	attacking prey from outside and killing them	generalists
Parasitoids:		
insects mainly from the orders Hymenoptera and Diptera, including important superfamilies Chalcidoidea and Ichneumonidea	depositing eggs in host; larvae exploit the host, which will finally die	specialists
Nematodes:		
from phylum Nematoda, important genera are *Steinernema* and *Heterorhabditis*	entering insect host via body openings, interaction with bacteria	generalists
Viruses:		
from different virus families, important families include *Baculoviridae* and *Parvoviridae* (densoviruses)	oral uptake by insect host, infection via gut epithel	specialists
Bacteria:		
from different bacterial families, important genera are *Bacillus*, *Serratia* and *Pseudomonas*	oral uptake by insect host, infection via gut epithel	generalists or specialists
Fungi:		
from different fungal phyla, most important are species from Ascomycota and Entomophthoromycota	infection through host cuticle or orally, growth in hemolymph	generalists or specialists
Microsporidia:		
unicellular pathogens, a sister group to fungi, an important genus is *Nosema*	oral uptake by insect host, infection via gut epithel	specialists

Types of natural enemies in insect production systems

Eilenberg et al 2017:

Natural enemies in insect production systems.

In Huis, A. (ed):
Insects as food and feed from production to consumption
Wageningen University Press, 201-222.

Insect pathogens

Obligate
Facultative
Potential
Specialists
Generalists

Insect virus can be responsible for collapse in production systems

A densovirus (AdDNV*)* frequently occurs in *Acheta domesticus*

A. domesticus AdDNV caused severe epidemics, with huge losses in production as a result. Replacement of *A. domesticus* by *Gryllus sigillatus* to avoid this virus disease

Replacement of *A. domesticus* could also be other members from the genus *Gryllus* (*Gryllus assimilis* and *Gryllus bimaculatus*) since they seem resistant to AdDNV

In the USA, such 'uncontrolled' introduction of new production cricket species have led to concerns

Liu et al (2011) Journal of Virology 85: 10069-10078.
Szelei at al (2011) Journal of Invertebrate Pathology 106: 394-399.
Weissmann et al (2012) Zootaxa 3504: 67-88.
Ballenger 2014 https://entomologytoday.org/tag/gryllus-locorojo/

Table 10.3. Insect viruses infecting crickets.

Virus name	Virus family (genome)	Cricket host species	Susceptible stages	Pathobiology	References
Acheta domesticus densovirus (AdDV)[1]	*Parvoviridae* (ssDNA)	*A. domesticus* *Gryllodus sigillatus* *Gryllus locorojo*	last nymphal stage, young adults	retarded grow, anorexia, lethargy followed by paralysation, finally death	Meynardier *et al.* (1977); Styer and Hamm (1991); Szelei *et al.* (2011); Weissman *et al.* (2012)
Cricket iridovirus (CrIV)[2]	*Iridoviridae* (dsDNA)	*A. domesticus* *Gryllus campestris* *Gryllus bimaculatus* *Gryllus assimilis* *Gryllus texensis*	nymphs and adults	reduced fecundity and life span, apathy and disorientation, death	Kleespies *et al.* (1999); Just and Essbauer (2001); Jakob *et al.* (2002); Adamo *et al.* (2014)
Cricket paralysis virus (CrPV)	*Dicistroviridae* (ssRNA)	*Teleogryllus oceanicus* *Teleogryllus commodus* *A. domesticus*	early- to mid-instar nymphs	paralysis followed by death	Reinganum *et al.* (1970, 1981); Morrissey and Edwards (1977); Scotti *et al.* (1981)
G. bimaculatus nudivirus (GbNV)	*Nudiviridae* (dsDNA)	*G. campestris* *G. bimaculatus* *T. oceanicus* *T. commodus*	nymphs (lethal), adults (chronic)	reduced grow, uncoordinated and lethargic behaviour, finally death	Huger (1985); Wang and Jehle (2009)
A. domesticus volvovirus (AdVVV)	Unassigned (ssDNA)	*A. domesticus* *G. assimilis*	unknown	unknown	Pham *et al.* (2013a,b)
A. domesticus mini ambidensovirus (AdMADV)	*Parvoviridae* (ssDNA)	*A. domesticus*	unknown	unknown	Pham *et al.* (2013c)

[1] Official species name *Orthopteran ambidensovirus 1* (www.ictvonline.org).
[2] Official species name *Invertebrate iridescent virus 6* (IIV-6) (www.ictvonline.org).

Eilenberg et al 2017: Natural enemies in insect production systems. In Huis, A. (ed):Insects as food and feed from production to consumption Wageningen University Press, 201-222.

First international survey on insect diseases in insects produced for food and feed

All information will be kept fully confidential with no references to companies or research teams, only anonymous summary tables will be used for the Wageningen conference May 2014 and possibly in a later publication

1) **Insect species produced (Latin and English name)**

……………………………………………………………………………………………………

2) **Symptoms on host**

……………………………………………………………………………………………………

……………………………………………………………………………………………………

3) **Effect on population of insects in the production (add an X)**

Severe reduction ☐ Some reduction ☐ No observed effect ☐

4) **How was the disease diagnosed (add an X)**

By yourself ☐ By an insect pathologist ☐ It was not diagnosed ☐

5) **Name (species or genus or group) of diagnosed insect pathogen (add an X)**

Virus ☐ Bacteria ☐ Fungi ☐ Microsporidia ☐ Other ☐

Name (if known) ……………………………………………………………………………

6) **Which actions were taken? If needed add several X**

Cleaning (soap etc) ☐ Sterilization ☐ Quarantine ☐ New breeding stock ☐

Notes about action and effect and if problem re-occurred

………

………

***Jørgen Eilenberg** (jei@plen.ku.dk) & **Annette Bruun Jensen** (abj@plen.ku.dk)*

University of Copenhagen, Denmark 2014

The 2014/2015 Survey

- First systematic attempt to collect information about insect diseases in production systems for food and feed
- A one page questionnary sent to companies and others who might have information
- Approx 30 responses

Selected results from survey

Production insect	Disease	Symptoms	Action
Acheta domesticus	Bacterium sp.	Increased mortality, red appearance	Cleaning of cages
Acheta domesticus	*Metarhizium* sp and *Beauveria bassiana*	Some mortality in population	Quarantine, new breeding stock
Acheta domesticus	Cricket paralysis virus CrPV	Collapse of cricket population, the virus seems to spread globally	Switching to new breeding stock or even new cricket species
Gryllus bimaculatus	*Gryllus bimaculatus* iridovirus	swollen abdomen, strikingly sluggish, mortality close to 100%	Occurs occasionally
Tenebrio molitor	*Beauveria bassiana*	Some mortality in population	Cleaning, removal of dead larvae, quarantine
Zophobas morio	*Pseudomonas* sp	Increased mortality, recurrent problem	Removal of dead larvae
Musca domestica	*Entomophthora muscae*	Dead adult flies with spores, epidemic	Cleaning, removal of dead flies, quarantine
Hermetia illucens	Unknown (Bacterium?)	Elongated and rounded mature larvae, moving slowly, then dying	Quarantine

Eilenberg et al, 2015, plus updates

Closed production facilities

A) **Closed production facilities** for lesser mealworm (*Alphitobius diaperinus*). Larvae are reared in plastic boxes on separate shelves. The Netherlands.

B) **Automatic separation** of *Tenebrio molitor* larvae from substrate. The Netherlands.

C) **Closed production facilities** for black soldier fly (*Hermetia illucens*). Larvae are reared in plastic boxes stacked at several meters height. South Africa.

Predators are of little significance.

Insect pathogen transmission within facility likely to occur.

Transmission of insect pathogens between facility and environment (via food, substrate, garbage) to some extent possible

Injuries caused by cannibalism or by physical handling likely to occur

Eilenberg & Jensen, book chapter in press   Photo credits. A, B: Proti-Farm, The Netherlands. C: Agri-Protein, South Africa.

Semi-open production facilities

D), E) **Semi-open production facilities** for house cricket (*Acheta domesticus)* and black cricket *Gryllus bimaculatus.* New cohorts are started in the different sections at time intervals. Thailand.

Predators (small and large) can be significant.

Transmission of insect pathogens within facility likely to occur. Transmission of insect pathogens between facility and environment (via air, insects, food, substrate, garbage) possible/likely to occur

Eilenberg & Jensen, book chapter in press

Photo credits: Afton Halloran, Univ. Copenhagen, Denmark.

Outdoor production facilities

K) **Outdoor hives** for honeybee (*Apis mellifera*) in almond plantation with low biological diversity. California, USA. L) **Outdoor hives** for *A* with medium biological diversity. Denmark. M) **Outdoor hives** for *A* with high biological diversity. Tanzania.

N) **Outdoor production/foraging** of Mopane moth *Gonimbrasia belina*, Zimbabwe

Predators and also parasitoids can be important

Transmission of insect pathogens within facility (hives, cells, trees) possible/likely to occur

Transmission of insect pathogens between facility and environment likely to occur (air, leaves, flowers, insects)

Eilenberg & Jensen, book chapter in press

Photo credits: Annette Bruun Jensen, Univ. Copenhagen, Denmark. http://www.nydailynews.com/life-style/eats/zimbabwe-favorite-snack-mopane-worms-article-1.1247669

A typical situation:

What is wrong with my *Tenebrio molitor* in culture ?

Some get discolored, some die

Is it an insect disease or some saprophytic microorganisms?

Any action needed?

Photo credits: E. Erin Morris, Univ. Copenhagen

Diagnosis needed

Bacterium, unknown species

Fungus (*Beauveria* sp.) infected

Nematode (*Steinermena feltiae*) infected

Uninfected

Photo credits: E. Erin Morris, Univ. Copenhagen

A case: Commercial product, Giant mealworms (*Zophobas morio*)

PhD student Gabriela Maciel-Vergara

- Recurrent problem: 2011-2012
- Weekly losses:10-20% weekly

- Current cleaning: potassium hydroxide & sodium hypochlorite
- Washing: 30-40^{0}C, disinfectant addition in last rinsing
- Early symptoms are detected in 4-6 weeks old larvae

Photo credits: Gabriela Maciel-Vergara

Initial diagnosis and observations:

- Cessation of feeding – curving body
- Larval cannibalism on diseased individuals
- Abnormal pupation and adult development (deformed wings & elytra)
- Dead insects – bad odour and melanization to some extent since first day after death
- No color changes besides melanization (gut disruption)
- Bacilli-like cells growth in hemocoel

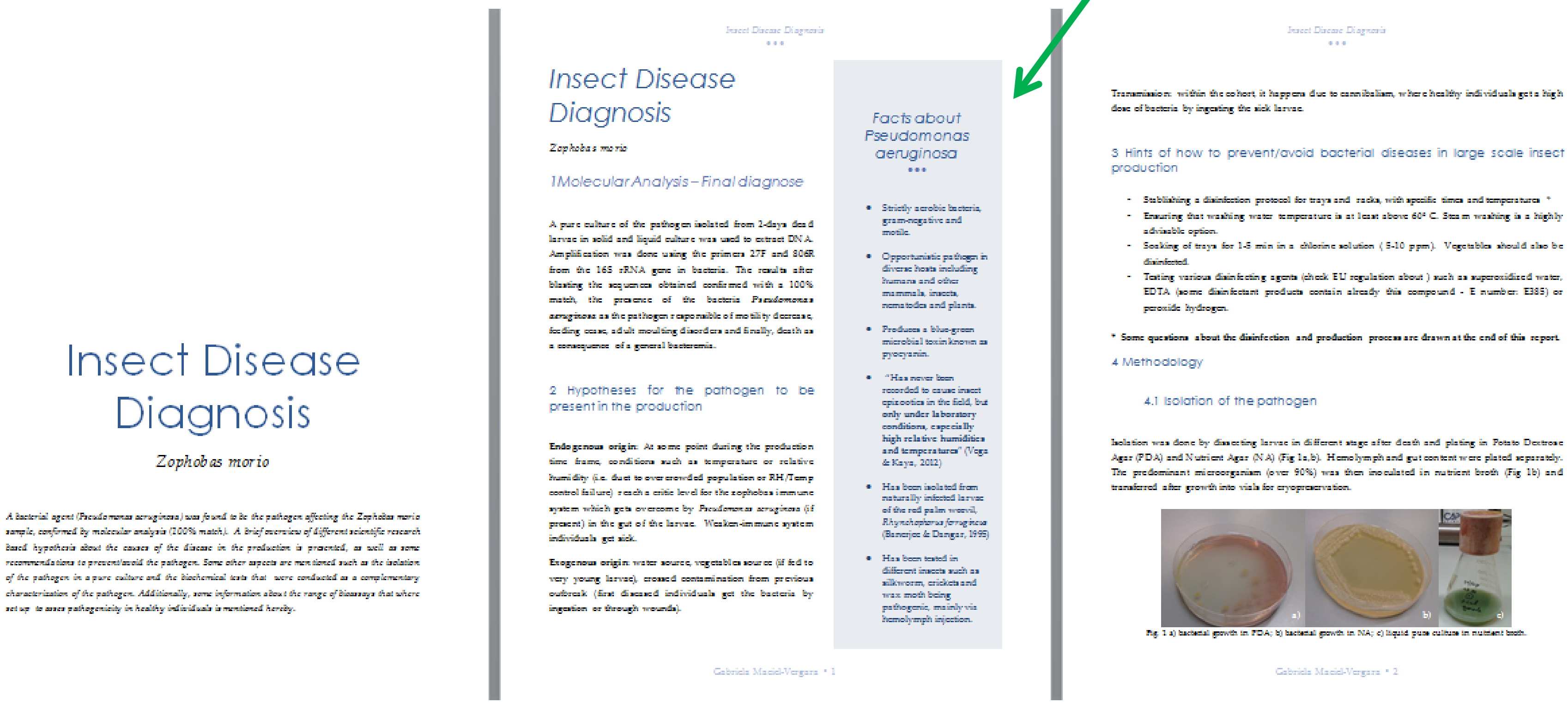
- Further characterization: pathogenicity, biochemistry, molecular

alive insects "curving"

larvae melanization:48 hrs after dead

Photo credits: Gabriela Maciel-Vergara

Information sheet for customer/industry



Insect Disease Diagnosis

Zophobas morio

A bacterial agent (Pseudomonas aeruginosa) was found to be the pathogen affecting the Zophobas morio sample, confirmed by molecular analysis (100% match). A brief overview of different scientific research based hypothesis about the causes of the disease in the production is presented, as well as some recommendations to prevent/avoid the pathogen. Some other aspects are mentioned such as the isolation of the pathogen in a pure culture and the biochemical tests that were conducted as a complementary characterization of the pathogen. Additionally, some information about the range of bioassays that where set up to asses pathogenicity in healthy individuals is mentioned hereby.

Insect Disease Diagnosis

Insect Disease Diagnosis

Zophobas morio

1 Molecular Analysis – Final diagnose

A pure culture of the pathogen isolated from 2-days dead larvae in solid and liquid culture was used to extract DNA. Amplification was done using the primers 27F and 806R from the 16S rRNA gene in bacteria. The results after blasting the sequences obtained confirmed with a 100% match, the presence of the bacteria *Pseudomonas aeruginosa* as the pathogen responsible of motility decrease, feeding cease, adult moulting disorders and finally, death as a consequence of a general bacteremia.

2 Hypotheses for the pathogen to be present in the production

Endogenous origin: At some point during the production time frame, conditions such as temperature or relative humidity (i.e. dust to overcrowded population or RH/Temp control failure) reach a critic level for the zophobas immune system which gets overcome by *Pseudomonas aeruginosa* (if present) in the gut of the larvae. Weaken-immune system individuals get sick.

Exogenous origin: water source, vegetables source (if fed to very young larvae), crossed contamination from previous outbreak (first diseased individuals get the bacteria by ingestion or through wounds).

Facts about Pseudomonas aeruginosa

- Strictly aerobic bacteria, gram-negative and motile.
- Opportunistic pathogen in diverse hosts including humans and other mammals, insects, nematodes and plants.
- Produces a blue-green microbial toxin known as pyocyanin.
- "Has never been recorded to cause insect epizootics in the field, but only under laboratory conditions, especially high relative humidities and temperatures" (Vega & Kaya, 2012)
- Has been isolated from naturally infected larvae of the red palm weevil, *Rhynchophorus ferrugineus* (Banerjee & Dangar, 1995)
- Has been tested in different insects such as silkworm, crickets and wax moth being pathogenic, mainly via hemolymph injection.

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Insect Disease Diagnosis

Transmission: within the cohort, it happens due to cannibalism, where healthy individuals get a high dose of bacteria by ingesting the sick larvae.

3 Hints of how to prevent/avoid bacterial diseases in large scale insect production

- Stablishing a disinfection protocol for trays and racks, with specific times and temperatures *
- Ensuring that washing water temperature is at least above 60° C. Steam washing is a highly advisable option.
- Soaking of trays for 1-5 min in a chlorine solution (5-10 ppm). Vegetables should also be disinfected.
- Testing various disinfecting agents (check EU regulation about) such as superoxidized water, EDTA (some disinfectant products contain already this compound - E number: E385) or peroxide hydrogen.

* Some questions about the disinfection and production process are drawn at the end of this report.

4 Methodology

4.1 Isolation of the pathogen

Isolation was done by dissecting larvae in different stage after death and plating in Potato Dextrose Agar (PDA) and Nutrient Agar (NA) (Fig 1a,b). Hemolymph and gut content were plated separately. The predominant microorganism (over 90%) was then inoculated in nutrient broth (Fig 1b) and transferred after growth into vials for cryopreservation.

Fig 1 a) bacterial growth in PDA; b) bacterial growth in NA; c) liquid pure culture in nutrient broth.

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<u>Overall sanitation process:</u>

- Disinfection protocol
- Diversity in disinfecting agents
- Disposition of discarded infected material

Photo credits: Gabriela Maciel-Vergara

What are probiotics?

Defined in 1965 as "**Growth promoting factors produced by microorganisms**." (Lilly and Stillwell 1965).

In 2001, the FAO/WHO referred to them as "**Live microorganisms which when administered in adequate amounts confer a health benefit to the host**"

Mealworms and bacteria

- **Screening and Identification**
  - Dissection of mealworm guts (focus on gut flora)
  - Culture on selective media for Lactic Acid Bacteria, ie. Lactobacillus and Bifidobacterium.
  - Identification to species level and candidate selection
- **Characterization**
  - Inhibition ability (antimicrobial activity)
  - Colonization potential
- **Formulation and In-vivo studies**
  - Delivery of probiotic bacteria
  - Storage stability

inVALUABLE

European collaboration 'INSECTPATH'

Develop a European service for **diagnosis of insect pathogens, other micro-organisms and non-infectious diseases** in production facilities of insects for food and feed (*Tenebrio*, *Atheta* etc) and foraged insects (*Formica* etc)

Collaboration with research teams on **insect production**, **insect pathology, food microbiology, animal husbandry, insect ethics and welfare**, in Europe and elsewhere

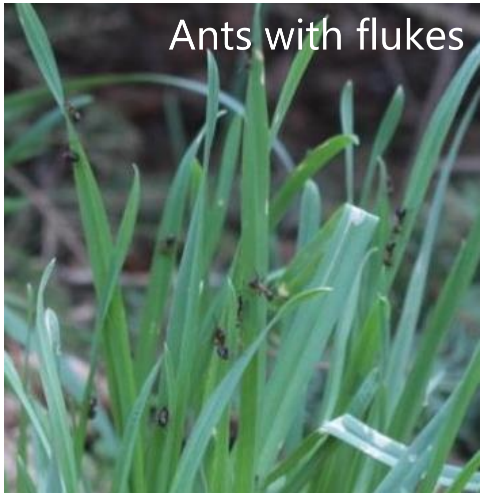
Collaboration with **companies**; **network activities** (for example via COST Actions); **Joint research** (national, Nordic, EU); **Teaching** (PhD level, MSc level)

Collaboration with EAAS (**European Federation of Animal Science**). New study commission 2016 about insects.

Collaboration with **similar services outside EU**. Dept. Entomol. at Mississippi State Univ., USA has an Insect Pathology Service running for decades, offering basic diagnosis of insect diseases. (http://www.irc.entomology.msstate.edu/service/)

Foraging, a case: Insects as intermediate host for vertebrate parasites

Formica ants are intermediate hosts of a fluke, **lancet liver fluke** (*Dicrocoelium dendriticum*)



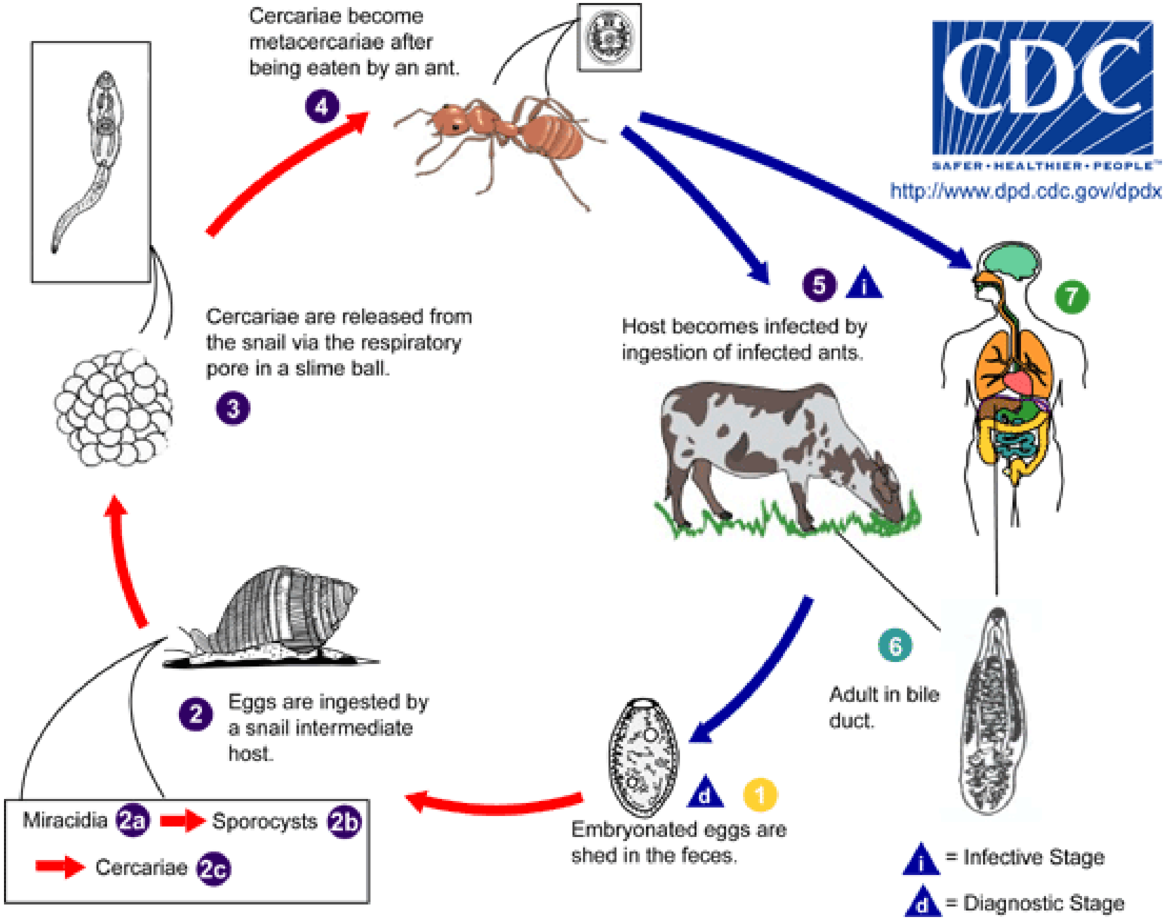


Photo credits: Joanna Malagocka, Univ. Copenhagen

Foraging, a case: Insects as intermediate host for vertebrate parasites

1) *Dicrocoelium dendriticum* (Phylum Platyhelminthes) fulfills the criterions for being regarded as an insect pathogen. For example, it manipulates the behaviour of infected ants

2) Studies on *D. dendriticum* may be undertaken from veterinary parasitology, food safety or insect pathology perspective

3) *D. dendriticum* metacercariae in host ants *Formica polyctena* are killed after exposure to different treatments of ants: freezing, boiling and ethanol. A.B. Jensen, J. Malagocka, B.L. Fredensborg, J . Eilenberg in prep for submission in September

4) **Advice: do not eat *Formica* ants raw, and be very cautious when eating other insects raw**

Harmful microorganisms as potential insect pathogens

Insects harbour many microorganisms. The microbial community in *T. molitor* larvae included *Propionibacterium, Haemophilius, Staphylococcus* and *Clostridium*. For *L. migratoria* genera *Weissella, Lactococcus* and *Yersinia* were common (Stoops et al 2015). Some of these microorganisms may act as potential insect pathogens, for example *Staphylocoocus aureus* infection in *T. molitor,* Dorling et al (2015).

Human pathogens may be carried by insects, although they do not seem to be insect pathogens*. Listeria innocua* did not colonize the gut of *T. molitor*, while it survived inside the gut upon freeze-drying (Vidal, 2015).

T. molitor has been shown to kill the human pathogen *S. aureus* within 30 min (Haine et al (2008).

Is there a possibility that more microorganisms are 'potential insect pathogens'

Dorling et al Developmental and Comparative Immunology (2014) 10.1016/j.dci.2014.08.010

(Stoops et al 2016) Food Microbiology 53: 122-127

Rosalía Flores Vidal, MSc thesis 2015: Survival of *Listeria innocua* inside *Tenebrio molitor* larvae's guts, University of Copenhagen

Haine et al (2008) Science 322: 1257-1259

Conclusions

- Insects in production systems have **many natural enemies**: predators, parasitoids, nematodes, fungi, bacteria, virus, microsporidia and more
- **Transmission of insect diseases** depends on production species and type of production facilities
- **Diagnostic methods** need further development
- Direct **collaboration with industry** to be strengthened
- **Probitics** may prove a new way to prevent insect diseases
- **Joint European initiatives** are being developed

Danish project partners

http://plen.ku.dk/english/research/organismal_biology/insect_pathology/insects-as-future-food-and-feed/

KU PLEN Staff on diseases in insects as food and feed: Annette Bruun Jensen, Antoine Lecocq, Gabriela V. de Maciel, Luna Santacoloma, Jørgen Eilenberg

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