MINIREVIEW

The greater wax moth Galleria mellonella: biology and use in immune studies

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ABSTRACT

The greater wax moth Galleria mellonella is an invertebrate that is increasingly being used in scientific research. Its ease of reproduction, numerous offspring, short development cycle, and finally, its known genome and immune-related transcriptome provide a convenient research model for investigation of insect immunity at biochemical and molecular levels. Galleria immunity, consisting of only innate mechanisms, shows adaptive plasticity, which has recently become the subject of intensive scientific research. This insect serves as a mini host in studies of the pathogenicity of microorganisms and in vivo tests of the effectiveness of single virulence factors as well as new antimicrobial compounds. Certainly, the Galleria mellonella species deserves our attention and appreciation for its contribution to the development of research on innate immune mechanisms. In this review article, we describe the biology of the greater wax moth, summarise the main advantages of using it as a model organism and present some of the main techniques facilitating work with this insect.

Keywords: Galleria mellonella; life history; insect model organism; insect immunity; host-pathogen interaction

INTRODUCTION

Recent advances in innate immunity studies have resulted in greater interest in Galleria mellonella as a model organism, which can be a source of the necessary tools required for investigations of conserved patterns of innate mechanisms in both vertebrates and other invertebrates. Despite the great scientific potential of insects, there is a need for more comprehensive publications describing the biology of chosen model organisms, working techniques and advantages of application thereof in immune studies. Many immunobiologists are often deprived of accessible sources of knowledge of the model organism that they work with. In our paper, we present an overview of the biological and scientific aspects of G. mellonella, a model organism that is increasingly being used in immunological and biomedical research. The sections of the paper present detailed biology of all developmental stages, a general overview of the immunity, scientific applications and some techniques associated with working on G. mellonella. We believe that the presented review, compiled largely based on our experience, will provide valuable information for both advanced and beginner scientists who have just started their scientific adventures with this undoubtedly great model organism, G. mellonella.
**BIOLOGY OF THE GREATER WAX MOTH G. MELLONELLA**

The greater wax moth was described for the first time in a colony of *Apis cerana* (eastern or Asiatic honeybee), that is, wild honeybees found in southern and eastern Asia. Its systematic position is presented in Fig. 1. Being a cosmopolitan species and pest of bee colonies, the greater wax moth has spread to almost all continents (except Antarctica), usually covering most or all of their areas (Kwadha *et al.* 2017). Its occurrence basically coincides with the beekeeping economy in individual countries, as this pest can be found in beehives or stored waxes causing a phenomenon called *galleriosis* (Fig. 2). According to the latest data, the greater wax moth has so far been confirmed in 27 countries in Africa, 9 in Asia, 5 in North America, 3 in Latin America, Australia and New Zealand and in 33 countries in Europe and almost all of the larger islands associated with them. It is expected that the species will continue to spread to unmanaged areas, which may be associated with changing climatic conditions (Kwadha *et al.* 2017; Pastagia and Patel 2007; Swamy 2008; Hosamani *et al.* 2017; Desai *et al.* 2019). About 4 days before eclosing, the larva is visible as a dark ring. Twelve hours before hatching, the fully formed larva is clearly visible through the thin chorion (Paddock 1918).

**Eggs**

Eggs, glued together, are laid in batches of 50 to 150 (Kwadha *et al.* 2017) or, as reported by Desai *et al.* (2019), even from 175 to 355. They are oval, white when laid and cream or pale pink when older. Reticulate and very rough, they are composed of interconnected polygons (squares, pentagons, hexagons and heptagons). The micropylar area is surrounded by concentrically arranged elements of the microstructure, reminiscent of rounded flower petals (Ellis, Graham and Mortensen 2013). The egg dimensions given by different authors are similar: length from 0.44 to 0.47 mm and width from 0.29 to 0.39 mm (Swamy 2008; Ellis, Graham and Mortensen 2013; Hosamani *et al.* 2017; Desai *et al.* 2019).

**Larvae**

Larvae most often hatch in the morning, between 08.30 and 11.00 h (Hosamani *et al.* 2017; Desai *et al.* 2019). Depending on the research carried out, egg survival ranges from ∼ 84 to 100% (Pastagia and Patel 2007; Swamy 2008; Hosamani *et al.* 2017; Desai *et al.* 2019). Shortly after hatching, larvae move from the cracks and crevices to the honeycomb, where they begin to feed and build protective silken tubes, destroying the honeycomb structure in the process. The directional movement and feeding are probably stimulated chemically. This was confirmed by Paddock (1918) and Nielsen and Brister (1979), who observed that *G. mellonella* larvae isolated from honeycombs always went back towards their food source. Feeding larvae usually expand their ever-widening tubes towards the central part of the honeycomb, where they tend to accumulate. In the absence of food, cannibalism may occur (Nielsen and Brister 1979; Williams 1997).

In natural conditions, *G. mellonella* larvae feed on honeycombs, which contain a significant amount of beeswax, some honey, exuviae of bee larvae and pollen residues. From such food, they obtain a large amount of energy but relatively little protein (Kwadha *et al.* 2017). If the amount of dietary protein falls below a certain level, the larvae cease spinning silk (Jindra and Sehnal 1989), probably due to the lack of essential amino acids for silk protein synthesis (Shaik, Mishra and Sehnal 2017). The protein content also affects the rate of larval development. Their growth is fast on old honeycombs, which contain bee maggots and pollen, but very slow on white or new honeycombs. The positive dietary effect of bee pollen on the growth rate of *G. mellonella* larvae and the fertility of females developing from them.
was confirmed by Mohamed et al. (2014). The rapid growth of foraging larvae leads to complete destruction of the honeycombs within a week of colonisation (Hosamani et al. 2017). Larvae can also develop on an artificial diet consisting of cereal products, milk powder, yeast, honey and glycerol (Desai et al. 2019). A close relationship between larval diet quality and resistance against pathogens has been demonstrated: if there is a deficiency of nutrients, larvae become susceptible to Candida albicans Berhout infection (Banville, Browne and Kavanagh 2012).

Feeding greater wax moth larvae spin protective silken tubes, within which they are not detected by bees (Shaik, Mishra and Sehnal 2017). However, host workers have been repeatedly observed removing dead larvae (presumably killed) of this pest (G. K. Wagner, oral information). This fact undoubtedly under-mines the 100% effectiveness of these silken structures to protect their owners. The composition of the silk from which the protective tubes are spun is similar to that in pupal cocoons. The core of the silk filament consists of heavy and light chain fibroins and the P25 chaperonin, whereas the filament coating is composed of sericins (Fediˇc, ˇZurovec and Sehnal 2002; Shaik, Mishra and Sehnal 2017). A feeding pause has been observed before each larval moult. Old cuticles are shed separately from the head capsule and the rest of the body. The average optimal larval development temperature for this moth is 29–33 °C (Warren and Huddleston 1962; Nielsen and Brister 1979; Williams 1997). The average duration of each consecutive larval instar L1-L7 is 4.08, 5.72, 5.28, 6.76, 7.64 and 8.40 days, respectively, giving a total duration of the larval stage of ~45 days (Pastagia and Patel 2007; Swamy 2008; Hosamani et al. 2017; Rahman et al. 2017; Desai et al. 2019). The last two larval instars grow the most intensively (Ellis, Graham and Mortensen 2013).

Immediately after eclosing, the first larval instar (L1) is white, slim and very short (mean length 1.27 mm) (Hosamani et al. 2017). During further growth, it turns greyish white in colour, and from the third larval stage onwards its body begins to thicken conspicuously, becoming massive and stocky by the end of its development (Fasasi and Malaka 2006; Ellis, Graham and Mortensen 2013; Kwadha et al. 2017; Desai et al. 2019). Being very weakly sclerotised, most of the body surface of the first-instar larva is devoid of pigment, except for the head (the most strongly sclerotised part of the body). In later larval instars, the tergites of the pronotum and abdominal segment X as well as the protarsus and claws of the ventral prolegs, which gradually darken after each moult, taking different shades from light to dark brown, are also well sclerotised (Ellis, Graham and Mortensen 2013). In the fully coloured final stage larva, a bright ecdysial line is visible along the middle of the dorsal side (especially well marked on the prothorax) (Kwadha et al. 2017; https://e-insects.wageningenacademic.com/galleria_mellonella).

The G. mellonella larva belongs to the polypod (eruciform or caterpillar-shaped) and peripneustic (nine pairs of spiracles) type. Its body consists of a head, a three-segmented thorax and an abdomen of 11 segments (Fig. 3). On the highly sclerotised head, there is a pair of short, two-segmented antennae, chewing mouthparts and four stemmata on each side – these are bright, oval and separated from each other (Ellis, Graham and Mortensen 2013). The presence of stemmata on the head of the G. mellonella larva is an important diagnostic character, which distinguishes the larva of this species from that of the lesser wax moth Achoa grisella, that is, another pyralid and apiary pest, which does not have this feature (Ellis, Graham and Mortensen 2013). The thorax bears three pairs of five-segmented thoracic legs (one pair per segment), each ending in a single hooked claw. There are prolegs on abdominal segments III–VI, which become visible 3 days after hatching (Desai et al. 2019). The terminal abdominal segment (XI) bears a pair of anal prolegs. There is one oval, brown and clearly visible spiracle on each side of the prothorax and on each side of abdominal segments I–VIII, a total of nine pairs (the peripneustic respiratory system); the last pair, on abdominal segment VIII, is the largest. The body bears rather thinly distributed, long, protruding, light brown, hair-like setae (Smith 1965; https://e-insects.wageningenacademic.com/galleria_mellonella, the present study).

Some authors report that there may be from 5 to as many as 10 larval instars in the development of G. mellonella. The smallest number (five) of larval stages has so far been reported by Fasasi and Malaka (2006), who explain that this quite unusual result is related to the type of food and other optimal conditions of their rearing programme, which required rapid development and thus a smaller number of molts. Other reports, however, including very recent ones, most frequently mention seven (L1–L7) larval instars in the development of this insect (Sehnal 1966; Anderson and Mignat 1970; Swamy 2008; Ellis, Graham and Mortensen 2013; Venkatesh Hosamani et al. 2017; Desai et al. 2019). This is confirmed by accurate measurements (in mm) of body length (l), body width (w) and head capsule width (wh) of L1-L7: 1.17, 1.27, 2.40, 4.80, 9.30, 15.50, 21.60 and 25.40, respectively; w-0.25, 0.45, 1.26,
1.56, 2.65, 3.30 and 4.86, respectively; wh-0.21, 0.32, 0.54, 1.15, 1.28, 1.55 and 2.30, respectively (Hosamani et al. 2017). In this context, the latest metric data regarding the length and width of the body of greater wax moth larvae, recently published by Desai et al. (2019), are worthy of attention: l-0.81, 2.10, 5.86, 8.76, 14.24, 19.58 and 23.88, respectively; w-0.29, 0.44, 1.11, 1.99, 2.03, 2.54 and 3.55, respectively. These figures differ conspicuously from those given 2 years before. This may have been caused by the different type of artificial food that was used for breeding; a mixture of wheat flour, corn flour, wheat bran, powdered milk, yeast, honey and glycerol. At the larval stage, there are still no external structural features enabling the sex of the future adult form to be determined (Kwadha et al. 2017).

Pupation

When fully grown, last instar larvae stop feeding and they move vigorously in search of suitable, safe places where they can attach the cocoon and pupate. In active beebees, these are mainly spaces beyond honeycombs (e.g. the outer surfaces of bee frames or the inner surfaces of the hive’s walls). In abandoned hives, by contrast, pupal cocoons have been found anywhere within them (G. K. Wagner, oral information). The wooden parts of the hive are often the sites where cocoons are constructed. Fully grown larvae excavate species-characteristic boat-shaped depressions in the wood, which can weaken the wooden parts of the hive (Paddock 1918; Ellis, Graham and Mortensen 2013). Having found and excavated a suitable site, the larvae begin to spin a silken pupal cocoon, which they then attach to the eroded cavities. Cocoon construction takes on average 2.25 days, although this depends on the abiotic conditions of the environment (Paddock 1918). The cocoon protects first the larva and then the pupa against worker bees and possible parasites and probably also stabilises the abiotic conditions during pupal development (Jinstra and Sehnal 1989; Shaik, Mishra and Sehnal 2017). The outer layer of the cocoon soon becomes hard while the interior remains soft (Ellis, Graham and Mortensen 2013). In the front of the cocoon, the larva makes an exit hole for the future adult. Just before pupation, however, this opening is closed off with a thin layer of silk (Paddock 1918; Desai et al. 2019). Having constructed the cocoon, the slightly shrunken larva becomes inactive a few hours before pupation, passing through a short-lived developmental stage known as a prepupa. As in all Lepidoptera, however, the G. mellonella prepupa is not considered to be of a distinct developmental stage because it is not separated from the last larval instar by a moult (Chapman 1998).

The entire developmental phase of the greater wax moth, in which the larva builds a cocoon and then pupates, has been defined as the preparatory period (Hosamani et al. 2017; Desai et al. 2019). During the pupal stage, as in other holometabolous insects, histolysis and phagocytosis of the larval structures take place first, followed by the histogenesis of the imaginary structures that arise from so-called imaginary disks. These are made from embryonic cells that can divide quickly. The whole process is controlled by hormones (Chapman 1998).

Pupa

Data on the external morphology of the G. mellonella pupa are given in relatively few reports (Paddock 1918; Smith 1965; Swamy 2008; Hosamani et al. 2017; Kwadha et al. 2017; Desai et al. 2019). Most often, these refer only to the general appearance of this developmental stage (e.g. colour, sexual dimorphism) and its dimensions. To date, only Smith (1965) has given a detailed account of the external structure of this pupa.

The pupa of the greater wax moth is obect (i.e. it represents a type in which all the appendages are cemented to the body by means of a special secretion). The colour of the pupa changes with age from white (just after pupation) through yellow and brown to dark brown 4 days later. The body is moderately elongate, −3.1–3.5-fold as long as wide in the widest place. The eyes are large and well visible. The antennae are long, slightly arched in the front, usually extending to the edge of the second pair of wings (hind wings). The pretarsus of the hind legs protrudes slightly beyond the edge of the hind wings (Smith 1965). There are two pairs of short, protruding setae on the paretials, resembling tiny horns. There are two to seven pairs of short setae on body segments. Segments II–VII are each equipped with a pair of active spiracles located on the sides of the body. The ventral side of abdominal segments VIII and IX exhibits well-marked sexual dimorphism: female – the sclerite of segment VIII is separated and segment IX has a single copulatory aperture; male – the sclerite of segment VIII is uniform and segment IX has a pair of rounded knobs representing the phallomeres and gonopore between them (Desai et al. 2019). The dimensions of the G. mellonella pupa given in the literature are: length: 11.9–20 mm; width: 3.2–7 mm (Paddock 1918; Smith 1965; Swamy 2008; Ellis, Graham and Mortensen 2013; Hosamani et al. 2017; Kwadha et al. 2017; Desai et al. 2019). The respective average dimensions of the female pupa are significantly larger than those of the male pupa: length−15.83 and 11.86 mm; width−4.17 and 3.17 mm (Desai et al. 2019).

Depending on the temperature and humidity, the pupal stage in G. mellonella lasts from 8 (at 28 °C, 65% RH - relative humidity) to ~50 days (from 2.5 °C to 24 °C, 44% to 100% RH) (Pastagia and Patel 2007; Swamy 2008; Hosamani et al. 2017; Kumar and Khan 2018; Desai et al. 2019).

Emergence of adults

The eclosion of adults from cocoons has been observed at night and late in the evening. As they leave the cocoons, they push out the silk lids covering the cocoon exit holes (Swamy 2008). Once free of the cocoons, the adults remain inactive until their wings are fully extended and hardened. At first, the moths are creamy white (teneral forms), later darkening to a grey colour (Nielsen and Brister 1979; Swamy 2008; Desai et al. 2019). It has frequently been observed that the imagines of G. mellonella prefer dark places, run around in an agitated manner if illuminated and try to hide in various unlit corners of the hive (G. K. Wagner, oral information).

Adults and mating

Adults are incapable of consuming food because their mouthparts are degenerate; hence, they do not live very long, from ~7 to 30 days, depending on ambient conditions (Paddock 1918; El-Sawaf 1950; Opoosun and Odebiyi 2009; Hosamani et al. 2017; Kumar and Khan 2018). As reported by El-Sawaf (1950), males live longer (21–30 days) than females (8–15 days), which have three phases in their lifetimes: pre-oviposition (1.60 ± 0.50 days), oviposition (6.12 ± 1.09 days) and post-oviposition (2.00 ± 0.87 days) (Desai et al. 2019).

Unlike most moths, G. mellonella adults have a unique mating behaviour. Males lure females with a two-component pheromone (n-nonanal + n-undecanal) and in addition emit short pulses of sound at a frequency of 75 kHz, which can play
a significant role in the selection of reproductive pairs (Finn and Payne 1977; Greenfield 1981). They generate this acoustic signal using structures found on the wings (Spangler, 1985, 1986). Females react to the sound by fanning their wings (Spangler 1988), although they are unable to locate its source. Sex pheromones, which are released by males in response to female wing movements, help in this, ultimately attracting their partners before mating (Leyrer and Monroe 1973; Spangler, 1985, 1986, 1987, 1988; Jones et al. 2002). Males begin to produce sound impulses after sunset, when the light intensity is near that inside the honey bee hive and they are close to or in contact with other wax moths. Interestingly, the sound is never produced in the presence of its natural hosts (i.e. honeybee workers; Spangler 1986). The exact mechanism of acoustic signal production in males of the greater wax moth was described by Spangler (1986). According to Nielsen and Brister (1977), copulation can take place on trees adjoining the apiary, after which only the females return to the hives.

**Oviposition and fertility**

Egg laying begins within a relatively short time after adults appear and mate (Paddock 1918). Nielsen and Brister (1977) observed oviposition ~24 h after the appearance of imagines, which continued for 4 consecutive nights. Females usually enter the hive at night, when the bees are already inactive. Attempts by G. mellonella females to get into the hives before evening have also been observed, but then they were attacked by aggressive host workers (Nielsen and Brister 1977). In the hive, the moths seek out various cracks and crevices on honeycombs or other parts of the hive (Charriere and Imdorf 1999), as far as possible from any light source. Having found a suitable place in the hive, the female stretches her abdomen to the maximum, extending the tip as deep as possible. The strategy described above minimises the detection of eggs by bees or possible parasites and increases the survival of the larvae hatched from them (Williams 1997; Ellis, Graham and Mortensen 2013; Kwadha et al. 2017). Hosamani et al. (2017) reports that oviposition usually takes place at night, between 19.00 and 03.00 h.

The overall fertility of G. mellonella females can differ widely: this is probably related to the abiotic and biotic conditions (including infections) in which they breed (Mohamed et al. 2014). The number of eggs laid by one female is usually from 500 to 1800 with ~66 eggs per day (El-Sawaf 1950; Warren and Huddleston 1962; Hosamani et al. 2017). Much smaller total numbers of eggs (i.e. from 107 to 297) were laid by single females in laboratory conditions (26.7°C, 93.0% RH) (Fasasi and Malaka 2006). Interesting data in this respect were obtained by Mohamed et al. (2014), who demonstrated a close relationship between various types of natural food and the fertility and duration of the oviposition period in G. mellonella females in constant breeding conditions (30°C, 50% RH). The lowest (392 eggs, 5.2 days) and highest (1308 eggs, 8.4 days) fertility and oviposition periods were obtained for females reared on an empty new wax comb and an old wax comb with pollen, respectively. As it turned out, however, the type of diet had only a minimal impact on the length of the embryonic development period, which ranged from ~10–11 days, depending on the type of food (Mohamed et al. 2014; Kumar and Khan 2018).

 Depending on the temperature, humidity and food resources, the overall developmental period from the oviposition to the appearance of adults ranges from ~32 days (28°C, 65% RH) to ~93 days (2.5–24°C, 44–100% RH, food shortage) (Kumar and Khan 2018). Because this moth usually lives in a fairly stable micro-environment (e.g. hive, warehouse) as regards prevailing abiotic conditions, it can periodically produce from four to six generations per year (Kwadha et al. 2017). Their number and longevity depend on environmental conditions, the most important of which appear to be the temperature and type of food (Mohamed et al. 2014; Kumar and Khan 2018).

**BIODEGRADATION OF POLYETHYLENE**

An amazing ability of G. mellonella larvae to digest polyethylene (i.e. one of the environmentally most burdensome and apparently non-biodegradable polymers) has been demonstrated (Bombelli, Howe and Bertocchini 2017). The authors became aware of this ability by accident, when holes appeared in the plastic bags in which they kept the caterpillars of this moth. It is believed that these unique abilities are related to its food preferences in natural conditions. The caterpillars normally feed only on honeycombs: these are made from beeswax, which contains a whole range of lipid compounds, including alkanes, alkenes, fatty acids and esters. As suggested by these researchers, biodegradation of these beeswax constituents probably requires breaking of the same kind of chemical bonds as those present in polyethylene. Greater wax moth larvae are not the only organisms capable of breaking down polyethylene, but they do so relatively quickly. It is yet to be ascertained, however, whether this activity of G. mellonella larvae in the digestion of hydrocarbons is due to the larva itself or to the enzymatic activity of its intestinal microflora. Currently, researchers are working on devising a simple and inexpensive technique for synthesising enzymes similar to those produced by greater wax moth caterpillars, which could be used on an industrial scale (Bombelli, Howe and Bertocchini 2017). Recently, Ren et al. (2019) reported that polyethylene could be degraded by Enterobacter sp. isolated from the gut of G. mellonella. In their future studies, the researchers are also planning to use four specific methods to potentially improve the degradation rate.

**G. MELLONELLA AS AN INSECT MODEL ORGANISM FOR IMMUNE STUDIES**

The larvae of the greater wax moth are broadly used in many aspects of immunological studies. This is because this insect meets most of the requirements for model organisms. First of all, its presence is not related to specific latitudes but is ubiquitous; hence, data obtained have general importance (Kwadha et al. 2017). Next, they can be reared easily and inexpensively in large amounts in the laboratory without any special equipment (Wojda 2017). The size of larvae allows precise injection of the required number of pathogens and sampling organs for further investigations: the hemolymph, hemocytes, fat body, trachea and gut. Finally, the relatively quick life span yields the next generations within a few months. G. mellonella is very useful for biochemical research since it is possible to easily obtain 20–40 μl of hemolymph from one larva, which pooled from a group of caterpillars can be further used for purification of bioactive molecules (Mak, Zdybicka-Barabas and Cytryńska 2010). Additionally, it is easy to make protein extracts from G. mellonella fat body, hemocytes and other organs. Furthermore, its usefulness for genetic research is on the increase. The entire transcriptome of immune-challenged larvae was obtained in 2011 and the entire genome was sequenced in 2018 (Vogel et al. 2011; Lange et al. 2018).
et al. 2018). This has opened new opportunities for identification of new bioactive molecules and for genetic manipulation. Finally, since the greater wax moth is a non-vertebrate model, there are no ethical formalities for its use as a mini-host.

An increase in the interest around insect immunity has been observed in recent years. It is probably caused by the greater awareness of the benefits of elucidation of the immune mechanisms in this group of animals. There are a few main reasons why scientists use insects in immune studies. These include: (i) analysis of the host-pathogen interaction; (ii) understanding of the innate immune mechanisms; (iii) testing the virulence factors of human pathogens; (iv) testing in vivo antimicrobial activity of new drugs; and (v) looking for bioactive molecules, for example, with antimicrobial, antiviral and anticancer activity and for use as biopesticides (Lionakis 2011; Tsai, Loh and Prof 2016; Pereira et al. 2018; Marrone 2019; Rossoni et al. 2019).

**G. mellonella in studies of host-pathogen interactions**

In the second part of the book *Alice in Wonderland* by Lewis Carroll, entitled *Through the Looking Glass*, Alice meets the Red Queen. They both run very fast; however, when Alice stops exhausted, she notices that they are still in the same place. Then the Red Queen explains to Alice: “Here, you see, it takes all the running you can do to keep in the same place”. This sentence was used by scientists to explain that all organisms have to adapt constantly to changing conditions to survive. In the area of immunobiology, it means that both pathogens and their hosts need to constantly improve their defence and virulence mechanisms, respectively, to avoid extinction. This is also known as the Red Queen Hypothesis or Evolutionary arm-races (Van Valen 1973; Joop and Vilincsiks 2016). In this context, the interaction of the greater wax moth with its natural pathogens can be studied.

*G. mellonella* is protected by chitin-containing integument. The cuticle also covers the internal organs of ectodermic origin (e.g. the trachea, foregut and hindgut), preventing the entry of the pathogens (Wojda 2017). When these barriers are broken, defence mechanisms are triggered. One of them is the activation of signalling pathways regulating the expression of antimicrobial peptides, which, when released to the hemolymph, act against the infecting microorganisms (Wojda et al. 2020). As suggested by their homology with *Drosophila melanogaster* peptides, they may belong to one of the families cecropins, diptericins, attacins, drosocins, defensins or metchnikowins (Hultmark 2003). After injury or infection, the phenol oxidase system is released from hemocytes (oenocytoides). It contains prophenol oxidase, which is pro-enzyme that needs to be digested by serine proteases to the active enzyme (PO). In parallel, serpins (inhibitors of serine proteases), which are part of the phenoloxidase complex, prevent enzyme hyperactivation, which can be dangerous to the host due to the release of free radicals upon melanin synthesis (Bidla et al. 2009; Demir, Gencer and Aylin 2012). This process relies on the synthesis of the dark melanin pigment from tyrosine catalysed by PO. Melanin can be deposited on the surface of pathogens, facilitating elimination thereof. Melanisation often accompanies the hemolymph coagulation process, making the clot harder, and prevents hemolymph efflux until the epidermis is restored (Li et al. 2002). It also may accompany the encapsulation process (cellular encapsulation) or formation of capsules without hemocyte compounds (humoral encapsulation). Both melanin synthesis and hemolymph coagulation are involved in immunity and wound healing (Eleftherianos and Revenis 2011). It is worth mentioning that coagulation in insects serves an important role in immunity, unlike in mammals, where blood coagulation is mainly engaged in wound healing. This is possible because the insect has an open circulatory system and coagulation as a defence process does not bring the risk of thrombosis, as is the case in animals with a closed circulatory system. Besides wound healing, coagulation may occur on the surface of intruding microorganisms (Eleftherianos and Revenis 2011).

In *G. mellonella*, lipophorins, including apolipophorin III, are the main coagulogens (proteins that are utilised during clot formation as a substrate to make an insoluble clot). These molecules together with anionic peptide-2 and lysozyme can be found in the hemolymph of non-immunised larvae (Mak, Zdybicka-Barabas and Cytryńska 2010). *G. mellonella* hemolymph appeared to be very rich in the 18-kDa apolipophorin III, which due to its multifunctionality can be called moonlighting protein or sometimes a “boisterous” protein. Besides its main function as a lipid transporter, it serves as PRR (pattern recognition receptor), neutralises endotoxins, enhances the activity of antibacterial molecules, and finally has antibacterial activity itself. It may also act as opsonine (Zdybicka-Barabas et al. 2012, 2015). Examples of humoral immune effector polypeptides from *G. mellonella* are summarised in Table 1.

The cellular branch of *Galleria* immunity is mediated by hemocytes. *G. mellonella* possesses five types of hemocytes: prohemocytes, granulocytes, plasmatocytes, oenocytes and spherulocytes. As mentioned above, the first two are adherent and able to engulf pathogens. Oenocytes contain propenoloxidase, which is released upon injury and infection, and spherulocytes transport cuticle components that cannot be synthesised in situ (Wojda 2017). In addition to phagocytosis performed by plasmatocytes and granulocytes, *G. mellonella* hemocytes can enclose groups of organisms in multicellular structures called nodules or bind big foreign bodies such as eggs of parasitic wasps in capsules. In such structures, pathogens cannot grow and can sometimes be killed by the lack of oxygen or by free radicals formed during melanin synthesis (i.e. a process often accompanying encapsulation; Wojda 2017).

Despite all the defence strategies mentioned above, natural pathogens are able to break the anatomical and physiological barriers, overcome the immune mechanisms and establish a biotope in the larval body where they can proliferate. For example, propagules of entomopathogenic fungi can attach to the insect cuticle and form an appressorium and a penetration peg, which due to its high turgor grows inside the cuticle and epidermis toward the hemocel. Besides mechanic pressure, the fungus secretes enzymes, such as lipases, chitinases and proteases, which digest insect tissue and hemolymph proteins, including defence molecules (Pendland, Hung and Boucias 1993; Tartar et al. 2005, Mondal et al. 2016). The fungi *Beauveria bassiana* and *Metharizium anisopliae* can naturally infect *G. mellonella*. There are two ways of infection of *G. mellonella* larvae in the laboratory, which are very close to the mode of infection in nature. The first method involves dipping the larvae in a specified concentration of fungal spores or placing them on a filter paper lining a funnel and washing with fungal spores. The other possibility is placing the larvae on a plate with a sporulating fungus (*Wojda, Kowalski and Jakubowicz 2009; Vertyporokh et al. 2015*). In both cases, fungal propagules attach to the insect cuticle and start infection. Some bacteria (e.g. *Bacillus thuringiensis*) can infect *G. mellonella* larvae through the oral route. When ingested, spores containing endotoxins (Cry, Cyt) bind to receptors present in the insect gut and perforate it (Soberon et al. 2009). Insects can die due to the intestinal damage or due to...
Table 1. G. mellonella humoral effectors.

| Proteins involved in microbial clearance | Proteins involved in melanisation, coagulation and wound healing |
|----------------------------------------|-------------------------------------------------|
| Anionic peptide-1<sup>1</sup>          | Alfa-crystallin<sup>6</sup>                     |
| Anionic peptide-2<sup>1</sup>          | Arylophorin<sup>7</sup>                          |
| Apolipophorin-III<sup>1,11</sup>      | Glutathione-S-transferase-like protein<sup>8</sup>|
| Cecropin A<sup>1</sup>                | Plasmatocyte spreading peptide<sup>9</sup>      |
| Cecropin D<sup>1</sup>                | Prophenoloxidase (Phenoloxidase)<sup>10</sup>   |
| Defensin<sup>1</sup>                  |                                                |
| Galiomycin<sup>3</sup>                |                                                |
| Gallysin<sup>1</sup>                  |                                                |
| Gloverin<sup>3</sup>                  |                                                |
| Heliocin-like peptide<sup>1</sup>     |                                                |
| Induced inhibitor of serine proteases-1<sup>2</sup> | Lipophorines<sup>11</sup>; Apolipophorin-I |
| Induced inhibitor of serine proteases-2<sup>2</sup> | Apolipophorin-II |
| Induced inhibitor of serine proteases-3<sup>2</sup> | Apolipophorin-III |
| Insect metalloprotease inhibitor<sup>3</sup> | Transglutaminase<sup>12</sup>                   |
| Lysozyme<sup>4</sup>                  |                                                |
| Moricin-like peptide A<sup>1,5</sup>  |                                                |
| Moricin-like peptide B<sup>1,5</sup>  |                                                |
| Moricin like peptide C<sub>4</sub>/C<sub>5</sub><sup>1,5</sup> |                                                |
| Moricin-like peptide C<sub>1</sub>/C<sub>2</sub>/C<sub>3</sub><sup>1,5</sup> |                                                |
| Moricin-like peptides D<sup>1,5</sup> |                                                |
| Proline-rich peptide-1<sup>1</sup>    |                                                |
| Proline-rich peptide-2<sup>1</sup>    |                                                |
| 6-tox protein<sup>1</sup>             |                                                |

Phipps, Chadwick and Aston 1994; Fröbius et al. 2000; Mak, Chmiel and Gacek 2001; Cytryńska et al. 2007; Wedde et al. 2007; Brown et al. 2009; Cytryńska 2009; Zdybicka-Barabas et al. 2012; Wu, Patocka and Kuc 2018

<sup>1</sup>antimicrobial peptide with direct antimicrobial activity.
<sup>2</sup>inhibitors of microbial enzymes.
<sup>3</sup>IMPI—the first peptidic specific inhibitor of metalloproteinases found in animals.
<sup>4</sup>may act non-enzymatically as AMP (antimicrobial peptide) and enzymatically (muramidase).
<sup>5</sup>found only in Lepidoptera, in some cases the protein products of different genes are the same.
<sup>6</sup>small heat shock protein, cellular coagulogen.
<sup>7</sup>hexamerin, humoral coagulogen.
<sup>8</sup>zymogen of phenol oxidase, catalysing melanin synthesis.
<sup>9</sup>enzyme catalysing formation of isopeptidic bonds during coagulation.

septicaemia when bacterial cells reach the hemocel through the perforated gut (Entwistle et al. 1993). It has been shown that toxins alone are not enough to kill G. mellonella, but entire spores are necessary, which indicates that the direct cause of their death is septicaemia rather than toxicaemia (Heimpel and Angus 1959; Li, Jarrett and Burges 1987). The oral route of infection can be used in the laboratory by addition of bacterial spores to natural or artificial diet. Another way is so-called force-feeding, when larvae are given a precise number of bacteria directly to their mouths (Ramarao, Nielsen-Leroux and Lereclus 2012). The modes of infection of G. mellonella larvae in the laboratory are shown in Fig. 4. After infection, relevant organs can be easily isolated for further investigation (Fig. 5).

In the body of infected G. mellonella, insect defence mechanisms are counteracted by the virulence strategies of the intruder: the one whose mechanisms are more effective will survive. In this antagonist co-evolution process, both species (the host and the pathogen) improve the defence and virulence mechanisms, respectively. Some of them are presented in Table 2.

One of the most impressive examples of the host-pathogen arms race found in G. mellonella is the interaction between the host’s antimicrobial peptides, protease inhibitors and the intruder’s extracellular proteases. Pathogens secrete proteases, which digest molecules of the infected host including antibacterial and antifungal proteins, thus preventing the host from effective defence (Cytryńska, Wojda and Jakubowicz 2016). Hence, despite the turned-on transcription of genes encoding AMPs followed by translation and secretion of defence molecules to the hemolymph, which are highly energy-consuming processes, the defence molecules cannot perform their function (Ortiz-Urquiza and Keyhani 2016). This pathogenic “tactic” is therefore aimed at the host’s defence mechanisms, giving the intruder an advantage in colonising the host. On the other hand, the action of extracellular proteases produced by the pathogen as virulence factors leads to formation of peptide fragments through the digestion of proteins and peptides. These short peptides, with a molecular weight below 3 kDa, which are called “protfrags”, serve as a “damage signal” and induce expression of genes encoding defence molecules (Griesch, Wedde and Vilcín-
Figure 4. Methods for experimental infection of G. mellonella larvae. Force feeding (A, B). The needle (capillary) needs to be inserted accurately and gently into the larval mouth hole without wounding the larvae and an exact volume must be introduced and ingested by the larva. Infection through the cuticle can be done by placing the larva on the filter paper and washing with a water-containing desired concentration of fungal spores (C and D) or by placing the larvae on a plate with sporulated fungus (for example, B. bassiana) and gently rolling (E). Microorganisms can also be introduced directly into the hemocel with the use of a needle introduced into the last or last-but-one proleg (F, G).

Among them, there are antimicrobial peptides and a specific inhibitor of metalloproteinase called the insect metalloproteinase inhibitor (IMPI). G. mellonella is the first animal organism in which a specific protein inhibitor of metalloproteinases has been found (Wedde et al. 1998, 2007). The IMPI protein is a thermostable ∼8.6 kDa protein, which does not have any homologue among all known inhibitors of metalloproteinases (Vilcinskas and Wedde 2002; Clermont et al. 2004). Its discovery opens new possibilities in the area of medical biotechnology, which will be discussed later.

Another vast area of investigations of evolutionary arms races, which is recently a very hot subject of research, is insect immune priming. This is the name of a phenomenon of increased resistance of individuals upon second exposure to the same (homologous priming) or a different (heterologous priming) pathogen (Cooper and Eleftherianos 2017; Wojda and Vertyporokh 2017). In other words, in contrast to classical immunology, which identifies the phenomenon of immunological memory with specific (acquired) immunity, the phenomenon of priming (or trained immunity in vertebrates) reflects the immunological memory within the mechanisms of innate immunity (Netea, Quintin and van der Meer 2011; Chambers and Schneider 2012). It is worth emphasising that there is no rule as to whether the first infection with a non-lethal dose of a pathogen will result in increased resistance upon the next infection of a given insect species. It appears that this may depend on energy resources (Contreras-Garduno et al. 2014). Recent research has demonstrated the phenomenon of immune priming in G. mellonella and presently there are studies trying to elucidate this phenomenon. For example, it has been shown that the immune response after surviving an infection with a non-lethal dose of B. thuringiensis is prolonged in comparison with animals meeting the pathogen for the first time (Taszkow, Vertyporokh and Wojda 2017). Similarly, increased activity was observed after reinfection of G. mellonella with the human opportunistic pathogen C. albicans (Vertyporokh et al. 2019). Increased expression of most of immune genes was observed in none of these studies, which indicates that enhanced efficiency of defence does not proceed through increased energy-consuming transcription but rather through more precise and more ergonomic immune response.

G. mellonella also serves as a useful model to study transgenerational immune priming (TGIP) including micro-host-pathogen co-evolution (Dubovskyi et al. 2013; Mukherjee et al. 2019). The main output from TGIP is that pathogens or their fragments can be deposited in laid eggs, which induces immunity in the offspring. However, there are cases where the offspring are more resistant when the father was infected, which cannot be explained in the same way when the mother was. Very interesting and reliable results come from the laboratory of Andreas Vilcinskas. The researchers show that pathogens can exert an effect on the expression of genes in the infected host, particularly in G. mellonella. These pathogen-induced epigenetic changes occur via histone acetylation and DNA methylation. DNA with acetylated histones and demethylated DNA are less condensed and the genes are more efficiently expressed, and vice versa, deacetylated histones and methylated DNA
### Table 2. Examples of an arms race between insect hosts and pathogens

| Possible host's strategies (insect) | Possible pathogen's strategies (bacteria or fungi) |
|-----------------------------------|--------------------------------------------------|
| Production of a sclerotic cuticle containing chitin, a layer of lipids and waxes that protect the insect against the entry of pathogens into the body (Klowden 2013) | Synthesis of cuticle-degrading enzymes: lipases, proteases and chitinases (Butt et al. 2016) |
| Recognition of the pathogen and activation of the immune response | Reduction of the number of pathogen-associated molecular patterns, which prevents the effective recognition, resulting in minimisation of the immune response (Wanchoo, Lewis and Keyhani 2009) |
| Coordinated reaction of the immune system, aimed at destroying pathogen cells | Destruction of host organs and tissues, especially those producing immune peptides, through the action of proteolytic enzymes |
| Synthesis of antimicrobial peptides | Secretion of proteases destroying antimicrobial peptides (Ortiz-Urquiza and Keyhani 2016) |
| Synthesis of protease inhibitors (Wedde et al. 1998, Fröbius et al. 2000) | Modification of the cell wall to hinder binding of antimicrobial peptides. Active removal of peptides from the cell (Joo, Fu and Otto 2016) |
| Synthesis of detoxification proteins (Vilcinskas et al. 1997) | Synthesis of toxic secondary metabolites (Ortiz-Urquiza and Keyhani 2016) |
| Phagocytosis and encapsulation | Avoidance of recognition by hemocytes (Pendland, Hung and Boucias 1993) |
| Possible enhanced resistance upon repeated contact with a given pathogen, ability to "remember" previous infections (Chambers and Schneider 2012) | Repeated infections |
| | What would the further pathogens’ answer be? |

make chromatin more compressed and hardly accessible for transcription factors (Vilcinskas 2016). Indeed, it appeared that changes in the activity of methylases/demethylases and acetylases/deacetylases were detected in infected G. mellonella larvae (Vilcinskas 2016).

**G. mellonella in studies of innate immunity and use as a mini host for human pathogens and in vivo testing of new drugs**

Insect immunity is called “evolutionary roots of human innate immunity” as it shares many features with human innate defence (Vilmos and Kurucz 1998; Sheehan et al. 2018). Since insects do not possess tools for classically understood acquired immunity (antibodies, memory T cells, major histocompatibility complex), their innate immune mechanisms in a pure form (not interfered with by acquired immunity) can be investigated (Buchmann 2014). A classical example is the discovery of Toll-like receptors in D. melanogaster, which greatly contributed to understanding the role of this receptor in the human immune system (Poltorak et al. 1998). This was awarded the Nobel Prize for Physiology and Medicine in 2011 “for discoveries concerning the activation of innate immunity” awarded to Bruce Beutler and Jules Hoffmann. In turn, the JAK/STAT pathway was first found in mammals and then it was found to regulate stress response and wound healing in insects (Stark and Darnell 2014).

The components of Toll and Imd pathways regulating the expression of AMPs are found in human cells as well. The insect Toll pathway has many common highly conservative components with the human Interleukin 1 pathway, while the insect Imd pathway resembles the human Interferon pathway (Hillyer 2016). In insects, signalling pathways regulate the expression of antimicrobial peptides. In turn, in mammals, they regulate the production of molecules regulating the activity of other immune cells (Hillyer 2016).

The other branch of innate immunity is cellular defence. G. mellonella adherent hemocytes (i.e. plasmatocytes and granulocytes) have some similarities with human macrophages and neutrophils; all of them are able to phagocytise foreign particles (Browne, Heelan and Kavanagh 2013).

Taking advantage of the fact that human pathogens may also cause insect death, their mutants can be used for testing their virulence (Tsai, Loh and Proft 2016). While searching for virulence factors, knockout, knockdown or overexpression of certain genes is performed and such mutant strains are checked for their virulence towards G. mellonella (examples: Harding et al. 2016).
that appeared in the first half of 2020 reporting the use of G. mellonella for in vivo investigations of the antibacterial and antifungal effect of compounds directed against human pathogens are presented in Table 3 (in vivo tests of only the toxicity of compounds are not included).

**G. mellonella in search of new bioactive molecules and natural pesticides**

Studying the immune response of G. mellonella and its interaction with natural pathogens (entomopathogens) may bring information of practical use. Natural pathogens such as bacteria B. thuringiensis or fungi B. bassiana and Metharhizium robertsi (anisopliae) are already used as biopesticides (Opisa et al. 2018). Crystal toxins of B. thuringiensis bind specifically to the insect gut; hence, they are selectively active against particular species without a lethal effect on others (Entwistle et al. 1993). Moreover, genes encoding toxins can be introduced into the plant genome, thus ensuring protection without application of bacteria or toxins. They have been successfully used for crop protection: potato, rice, maize and cotton (Clark, Phillips and Coats 2005; Meho et al. 2005).

There are also approaches to express insect defence molecules in crops to increase their resistance to bacterial or fungal pathogens. For example, it has been shown that insect cecropins inhibit the growth of bacterial and fungal phytopathogens (Cavallarin, Andreu and Segundo 1998; Sharma et al. 2000). Transgenic expression of cecropin B from Bombyx mori in rice resulted in enhanced resistance to bacterial blight (Sharma et al. 2000). Expression of cecropin B analogues in tobacco enhanced the resistance to Pseudomonas solanacearum caused wilt (Jaynes et al. 1993). Also, attacines from Lepidoptera expressed in apple and pear confer resistance to a bacterial disease called fire blight (Reynoard et al. 1999; Ko et al. 2000). Great hopes are also attached to antifungal peptides, namely defensins. Transgenic expression of heliomycin in tobacco increases the resistance of these plants to fungal infection (Banjet et al. 2002). Finally, gallerimycin from G. mellonella expressed in tobacco using Agrobacterium tumefaciens as a vector conferred resistance to the fungal pathogens Erysiphe cichoracearum and Sclerotinia minor (Langen et al. 2006). Moreover, derivatives of proteases from silk produced by G. mellonella (SPI2) transformed with the use of A. tumefaciens to potatoes increased their in vitro resistance to late blight Phytophthora infestans, being harmless to non-target organisms (Kodrik et al. 2013).

It is worth mentioning that some approaches are undertaken to reduce the proteolytic degradation of defence molecules in transgenic plants, which is one of the main problems encountered. Also, to avoid reduction in the fitness of plants by constitutive expression of insect defence molecules, there are successful attempts to express insect polypeptides from promoters induced after infection (for the review see Vilcinskas and Gross 2005).

Besides agriculture, there is great hope to use insect molecules in the fight against human pathogens and cancer cells. For many years, insect antimicrobial peptides have been expected to complement antibiotics for treatment of infectious diseases, because they do not cause resistance in microorganisms. Recently, it has been reported that G. mellonella cecropin A exhibits activity against uropathogenic E. coli, including biofilm eradication (Kalsy et al. 2020). Additionally, AMPs of insect origin can be synthesised chemically and tested for their antiviral and...
Table 3. The use of G. mellonella for in vivo investigations of compounds against human pathogens: reports published in the first half of 2020.

| Anti-inflammatory compounds | Antimicrobial compounds | Antifungal compounds |
|----------------------------|------------------------|----------------------|
| Liposomal nanoparticles incorporating anidulafungin against C. albicans (Vera-González et al. 2020) | Itraconazole against Scutiger brasiensis and Candida albicans (Passos et al. 2020) | Kyotorphins against C. albicans (De Andrade et al. 2020) |
| Ribavirin alone and in combination with fluconazole against C. albicans (Zhang et al. 2020) | Caspofungin, fluphenazine and their combination against Candida glabrata (Garzon et al. 2020) | Lactoferrin and amphotericin B against C. albicans and C. neoformans (Fernandes et al. 2020) |
| Activity of copper(I), manganese(I) and silver(I) 1,10-phenanthroline chelates against Candida haemuloni (Gandra et al. 2020) | Polyketides against S. aureus infection (Loges et al. 2020) | EeCentrocin 1 derived peptide EC1-17KV against C. albicans (Ma et al. 2020) |
| Minocycline-azole combinations against pathogenic fungi including Aspergillus fumigatus, Aspergillus flavus, Exophiala dermatitidis, Fusarium solani and Fusarium oxysporum (Gao et al. 2020) | Micocycline in combination with fluconazole against fluconazole-resistant Cryptococcus neoformans (Kong et al. 2020) | 4-chloro-3-nitrophenyldifluorodiaryl methane against C. albicans (Staniszewska et al. 2020) |
| Micocycline in combination with micocycline against C. neoformans (De Castro Spadari et al. 2020) | Voriconazole against cryptococcosis infections (De Castro Spadari et al. 2020) |

The discovery of the specific inhibitor of metalloproteinases IMPI mentioned above has raised hopes of using it as a drug against human pathogens. Many of them secrete extracellular metalloproteinases as virulence factors involved in overcoming the anatomical barriers and destroying insect defence proteins and peptides (Monod 2008; Benitez and Silva 2016; Eisenhardt et al. 2019). The use of peptide inhibitors directed against extracellular metalloproteinases would probably ease treatment of infections caused by bacterial and fungal pathogens.

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The reader with this interesting insect species regarding its biology, behaviour and curiosities and as an insect model organism that is increasingly being used in laboratories around the world.

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