Standardized methods to rear high-quality Galleria mellonella larvae for the study of fungal pathogens


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INTRODUCTION

Reliable animal models remain essential to study the pathogenesis of infectious diseases. Mammalian models are popular due to their similar physiology to humans, but their use faces ethical, logistical, and financial issues. Invertebrate hosts are an excellent alternative, especially for large-scale pathogenesis studies, because of their limited ethical constraints (Glavis-Bloom et al., 2012). The nematode Caenorhabditis elegans (Maupas) and the fruit fly Drosophila melanogaster Meigen are two of the best-established invertebrate models. Nevertheless, their use for the study of human pathogens is restricted. For example, both C. elegans and D. melanogaster cannot survive at mammalian temperatures, and their small size makes it hard to inoculate a precise quantity of pathogen (Desalermos et al., 2012). To this end, the greater wax moth, Galleria mellonella (L.) (Lepidoptera: Pyralidae), has gathered widespread interest in the biomedical research community as an alternative non-mammalian model to study bacterial and fungal infections in humans. Nevertheless, most laboratories rely on commercial breeding companies that grow these larvae in bulk. Variation in rearing conditions between these companies and shipping of the larvae affects the response of G. mellonella to infection, both decreasing the reproducibility and comparability of infection experiments across laboratories. To unlock the full potential of G. mellonella as an infection model, standardized rearing is essential to produce healthy larvae of uniform quality. To this end, we developed an optimized protocol using a high-nutritional artificial diet and cost-effective housing solutions to ensure optimal conditions during each stage of the G. mellonella life cycle. This resulted in the rapid generation of larvae with a short larval phase, large size, and increased weight compared to other protocols and commercial larvae. A survival analysis comparing the standardized larvae with commercially obtained larvae showed significantly higher survival of standardized reared larvae after infection with Candida albicans (CP Robin) Berkhout (Saccharomycetales), proving the higher quality of standardized reared larvae.

KEYWORDS

biomedical research, Candida albicans, Galleria mellonella, greater wax moth, infection model, Lepidoptera, non-mammalian model, pathogenesis, Pyralidae, rearing, standardization
TABLE 1  Comparison of experimental conditions used by different laboratories for Candida albicans infection studies in Galleria mellonella

<table>
<thead>
<tr>
<th>C. albicans strain</th>
<th>Larval size (mg)*</th>
<th>Supplier</th>
<th>Storage conditions</th>
<th>Infection dose (CFU/larvae)</th>
<th>Incubation period (days)</th>
<th>Mortality (%)b</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 8758; SC5314</td>
<td>300 ± 30</td>
<td>Allevamento Cirà, Como, Italy</td>
<td>15 °C, wood shavings, dark, 7 days</td>
<td>5 × 10^5</td>
<td>9</td>
<td>100 (3)</td>
<td>Ottaviano et al., 2021</td>
</tr>
<tr>
<td>200–300</td>
<td>ACHETA Farm, Martinýes, Czech Republic</td>
<td>18 °C, wood shavings, dark, 7 days</td>
<td>2 × 10^5</td>
<td>9</td>
<td>100 (6)</td>
<td>Dekkerová-Chupáková et al., 2018</td>
<td></td>
</tr>
<tr>
<td>275–330</td>
<td>Sciencia, Cali, Colombia</td>
<td>4 °C, dark</td>
<td>5 × 10^6</td>
<td>10</td>
<td>100 (2)</td>
<td>Muñoz et al., 2020</td>
<td></td>
</tr>
<tr>
<td>CBS 6431; ATCC 10231</td>
<td>200–400</td>
<td>MealWorm Company, Sheffield, UK</td>
<td>15 °C, wood shavings, dark, 21 days</td>
<td>2 × 10^6</td>
<td>3</td>
<td>20</td>
<td>Cotter et al., 2000</td>
</tr>
<tr>
<td>200–250</td>
<td>Tianjin Huiyu Biotech., Tianjin, China</td>
<td>12 °C, dark, 14 days</td>
<td>5 × 10^6</td>
<td>4</td>
<td>90</td>
<td>Su et al., 2020</td>
<td></td>
</tr>
<tr>
<td>250–350</td>
<td>BioSystems Technology, Devon, UK</td>
<td>15 °C</td>
<td>5 × 10^5</td>
<td>5</td>
<td>10</td>
<td>Romera et al., 2020</td>
<td></td>
</tr>
<tr>
<td>CBS 562; ATCC 18804</td>
<td>250 ± 50</td>
<td>Alcotán, Valencia, Spain</td>
<td>Not mentioned</td>
<td>5 × 10^5</td>
<td>7</td>
<td>100 (3)</td>
<td>Frenkel et al., 2016</td>
</tr>
<tr>
<td>Clinical isolates</td>
<td>300 ± 20</td>
<td>Bichosa live bait, Salceda de Caselas, Spain</td>
<td>15 °C, wood shavings, dark, 7 days</td>
<td>5 × 10^5</td>
<td>7</td>
<td>80–100 (2)</td>
<td>Marcos-Zambrano et al., 2020</td>
</tr>
<tr>
<td>330 ± 25</td>
<td>Vanderhorst, St. Marys, OH, USA</td>
<td>Dark, 7 days</td>
<td>10^5</td>
<td>4</td>
<td>100 (1–4)</td>
<td>Junqueira et al., 2011</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>Livefood UK, Rooks Bridge, UK</td>
<td>RT, dark, 2 days</td>
<td>10^6</td>
<td>Ca. 3</td>
<td>100 (2)</td>
<td>Borman et al., 2016</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD.

b In parentheses, the number of days within the indicated incubation period needed to reach 100% mortality.

RT, room temperature.
MATERIAL AND METHODS

Larvae

Larvae were originally obtained from Kreca Ento-Feed (Ermelo, The Netherlands). This company maintains its own greater wax moth culture without addition of antimicrobial compounds or hormones to the artificial diet. The larvae varied naturally from cream-colored to ash-grey (see Figure 1). As most larvae showed an intermediate light-grey pigmentation these were used for experiments (Figure 1A2).

Housing materials

Jars made of hard plastic (approximately 12.5 cm high and 11 cm in diameter) were used for rearing the larvae (step 2 in Figure 2). Jar lids were modified by cutting a large hole through the center and replacing it with ultra-thin stainless-steel wire mesh (sieve size of 0.1 mm²) to allow ventilation and prevent small caterpillars from getting out. When larvae started to spin cocoons and reached the pupal stage, the jars were placed in a hard plastic container without the lids to give the larvae more space for pupation and to allow better ventilation to prevent molding and the emergence of healthy moths. The container fitted at least four jars and ventilation holes were made at opposite sides of the container again covered with stainless-steel wire mesh (step 5 in Figure 2). Emerged moths were transferred to a closed non-transparent bucket in which holes were made that were covered by filter paper on which eggs were laid, enabling easy transfer of eggs to new jars (step 6 in Figure 2). After each generation, all materials were cleaned with soap and 70% ethanol before using them again.

Artificial diet

The composition of the artificial diet was based on an optimized diet for *G. mellonella* described by Hickin.
et al. (2021). Table 2 gives the ingredients for this diet of high nutritional quality. To prepare the diet, all dry ingredients, including the beeswax pellets, were weighed and mixed in a closed container. The liquid ingredients were combined in a separate beaker and the mixture was heated in a microwave until lukewarm. After mixing with a spatula, the hot mixture was added to the dry ingredients and thoroughly stirred until no dry components were visible anymore. The artificial diet was allowed to cool down and stored in a closed container at 4 °C for up to 2 months.

Protocol for rearing

We established a protocol for the standardized breeding of G. mellonella (Figure 2). The complete life cycle of G. mellonella from egg to moth takes approximately 8–13 weeks, depending mostly on temperature and diet composition (Jorjão et al., 2018; Desai et al., 2019; Firacative et al., 2020; Wojda et al., 2020). Under the conditions described below, the observed life cycle from egg to moth was about 8 weeks. All rearing steps took place at 25 °C and 50–60% r.h. The steps are as follows (for an overview of every step see Figure 2):

1. Eggs were collected on filter paper every 2 days until moths started to die and egg laying stopped. One circle of eggs (see Figure 2, step 1) usually contained 500–1000 eggs.
2. The filter paper was cut, and one or two egg circles were transferred to the housing jars together with 50 g of diet. The jars were stored at L14:D10 photoperiod and checked once a week to monitor caterpillar growth. After about 7–10 days, eggs started to hatch and the first larvae appeared.
3. In the next 2–3 weeks, larvae fed on the medium and developed into mature larvae. About 14 days after hatching, larvae reached a length of 1 cm (second or third instar) and started to produce high amounts of webbing/silk. At this point larvae also started to grow exponentially. To avoid overcrowding of the jars, larvae were divided by size – small: 0.5–1 cm, medium: 1–2 cm, and large: >2 cm – and placed into clean jars filled with fresh diet. This process was repeated at least twice a week.

**Note:** Ample diet should be available at all times during this phase to enable further larval development. Larvae that starve for longer periods will stop growing and go into the pre-pupal phase.

4. Healthy looking larvae without pigmentation of about 3 cm long and 300–400 mg weight were used for experiments. When webbing is removed daily, and fresh diet is given to prevent starvation, larvae may stay in this instar for several weeks (also mentioned by Pereira & Rossi, 2020). If used within 5 days after reaching 300–400 mg, we could monitor the larvae for at least 10 days during experiments, without transition to the pupal stage.

**Note:** Larval color varies naturally from creamy white to ash grey (Desai et al., 2019; Dubovskiy et al., 2013). Therefore, equal greyish pigmentation of the dorsal side is not a sign of disease. The ventral side of the larvae should be creamy white and without any grey markings (Figure 2). Nonetheless, it is best to use larvae of equal coloration, as fungal infection may cause different survival rates between white and grey larvae (Dubovskiy et al., 2013).

5. About 5–6 weeks after hatching, larvae stopped feeding and started spinning cocoons. After the first larvae start pupation, it is common for all larvae to reach the pupal state within a few days (Pereira & Rossi, 2020). Therefore, jars containing pupae should be separated from jars containing earlier stage larvae. To this end, the lids were removed from jars with pupating larvae and these jars were isolated in a bigger container. Some diet was left in the jars for smaller larvae that are still feeding.

**Note:** Earlier instars also spin cocoons, but these are thin and easy to remove. At the pre-pupal stage cocoons are more rigid and hard to remove. At this point, larvae enter their inactive (pupal) state and should not be used in experiments anymore, as this could cause bias in survival rates.

6. In about 2 weeks after pupation, emerged moths in the plastic containers were anesthetized with CO₂ and 50–200 moths were transferred to closed non-transparent buckets, which were prepared to support egg laying by covering the ventilation holes with filter paper on which moths can lay their eggs.

**Note:** No standard ratio of female-to-male moths is needed, as male moths can fertilize multiple females (Jorjão et al., 2018).

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**TABLE 2** Composition of the optimized Galleria mellonella diet

<table>
<thead>
<tr>
<th>Diet ingredient</th>
<th>Amount (g)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice flour</td>
<td>54</td>
<td>9.5</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>54</td>
<td>9.5</td>
</tr>
<tr>
<td>Wheatgerm</td>
<td>28</td>
<td>5.0</td>
</tr>
<tr>
<td>Torula yeast</td>
<td>84</td>
<td>14.8</td>
</tr>
<tr>
<td>Beeswax</td>
<td>22</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Liquid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Honey</td>
<td>136</td>
<td>24.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>128</td>
<td>22.6</td>
</tr>
<tr>
<td>Water</td>
<td>60</td>
<td>10.7</td>
</tr>
</tbody>
</table>
Comparison between commercial and standardized larvae

To assess whether standardization of the G. mellonella rearing process leads to healthier larvae, the survival of standardized reared larvae was compared with that of commercially bred larvae purchased from Kreca Ento-Feed (Ermelo, The Netherlands). To this end, a survival assay was carried out using two C. albicans reference strains (CBS562 and CBS6431), which were previously shown to be virulent in G. mellonella (Frenkel et al., 2016; Cotter et al., 2000; Romera et al., 2020; Su et al., 2020). In a dose–response assay, a range of inoculum concentrations of both strains was injected to groups of larvae, to determine how many cells are needed to kill at least 50% of the larvae (LD50). Before the start of the experiments, fungal strains were grown overnight in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) at 200 rpm and 30 °C. For both commercial and standardized G. mellonella, the largest larvae without any dark dispersed pigmentation, but with light grey coloration (see Figure 1A2), were selected and weighed to have uniform groups of larvae. Fungal inocula were prepared and groups of 15 larvae were inoculated by intra-hemocoel injection at the site of the last pro-leg with the use of a 26-gauge Hamilton syringe as described by Fuchs et al. (2010). Two control groups were included: untreated larvae and larvae injected with phosphate-buffered saline (PBS; without Ca, Mg; pH 7.3–7.5; Lonza, Basel, Switzerland). Larvae were placed in Petri dishes without food and incubated at 37 °C over a period of 10 days. Survival was checked daily, and dead larvae were removed together with webbing and feces. Experiments were always performed in triplicate.

Statistical analysis

The results were analyzed using IBM SPSS Statistics v.27 software (IBM, Armonk, NY, USA). An independent samples t-test was used to compare larval weights among treatments. Kaplan–Meier survival plots were generated and analyzed using the Mantel-Cox Log-rank test.

RESULTS AND DISCUSSION

So far, most rearing protocols focused on optimizing the diet (Jorjão et al., 2018; Hickin et al., 2021) or housing conditions (Firacative et al., 2020; Pereira & Rossi, 2020). Our optimized protocol unifies these methods. The various housing containers ensured easy collection of the eggs, optimal conditions for each stage of the G. mellonella life cycle, and lower risk of infections that could destroy the...
whole colony. Together with a high-nutritional diet, this resulted in a fast life cycle, with larvae already reaching a weight of >300 mg after about 26 days (starting from the egg). This is, thus, much faster than the approximately 40 days generally reported for larvae to reach this size. Moreover, with 6 weeks instead of 8, the development from egg to mature moths was also about 2 weeks shorter (Jorjão et al., 2018; Desai et al., 2019; Firacative et al., 2020; Pereira & Rossi, 2020). A short larval phase reduces the time needed to obtain larvae with the right size for experiments and also generates healthier larvae (Jorjão et al., 2018).

Comparison between the standardized reared larvae and commercially obtained larvae confirmed that standardized larvae could withstand higher fungal infection loads (Figure 3). The LD<sub>50</sub> of both C. albicans reference strains tested was twice as high for standardized larvae (10<sup>6</sup> vs. 5 x 10<sup>5</sup> CFU for commercial larvae) (Figure 3). This increased resistance to infections could mean survival curves are not suitable anymore for fungal strains or species that already require a very high inoculum to kill the larvae. However, there are other health scoring systems that could be applied to score the response to infection with this kind of low virulent pathogens. For example, the activity of the larvae, the degree of melanization, and cocoon formation (Champion et al., 2018).

Standardized larvae did not show any deaths in the control groups, where commercial larvae occasionally died (Figure 3), which is important for the reproducibility of the experiments. In addition to the difference in results of the survival assay, the commercially obtained larvae were highly variable in quality. This is not reflected in the survival assay, as only healthy active larvae were used in this assay. Even though the local supplier maintains its own colony of G. mellonella without the use of hormones or antimicrobials, larvae often varied in size, showed lots of pigmentation, or just died spontaneously. Such variability decreases experimental reproducibility and is detrimental for the continuity of experiments, strengthening the need for standardized in-house bred larvae.

The variable quality of commercially grown larvae could be caused by many factors (Champion et al. 2018; Pereira & Rossi 2020). Especially the availability of food and the diet composition seem to have significant effects on the G. mellonella immune system. Starvation is a common pre-treatment, also because commercial larvae are often shipped and stored on wood shavings without any nutritional value. Food deprivation during larval development leads to a reduction in cellular immune responses resulting in increased susceptibility to infections (Banville et al., 2012). Therefore, larvae should be reared and stored on a suitable diet, even when ordered. Although Krams et al. (2015) reported that low-energy diets increase the encapsulation response of G. mellonella, these authors also observed slower larval development, reduced body mass, and decreased survival rates compared to larvae fed a high-energy diet. A suitable diet consisting of various nutrients, such as carbohydrates, proteins, and lipids activates the immune response of G. mellonella (Jorjão et al., 2018). With the diet being such an important factor for larval quality, standardization is crucial to allow inter-laboratory comparisons. Therefore, our protocol incorporates the optimized diet described by Hickin et al. (2021) that supports high survival and greater larval body mass. This was also reflected by our results, as larvae fed this diet reached a higher body mass than commercial larvae (mean ± SD = 364 ± 56 vs. 239 ± 51 mg; t = −9.100, d.f. =58, P < 0.001). After preparation, the diet can be stored in a closed container for at least 2 months, unlike other diets that need to be prepared fresh (Firacative et al., 2020).

Other factors, such as humidity and temperature, have also been shown to affect the G. mellonella life cycle and susceptibility to infection (Pereira & Rossi 2020). In general, G. mellonella is maintained at a temperature range of 25–37 °C. Relative humidity levels should not get below 33% as this decreases adult longevity and fecundity and reduces the moths’ ability to lay eggs (Hanumanthaswamy et al., 2013). Light exposure is also reported to influence larval development. Constant illumination slows down the larval metamorphosis and reduces the final weight (Kryspin et al., 1974). Nevertheless, under a L12:D12 photoperiod the effects of light are less profound. Compared to larvae kept in constant darkness, larvae reared under L12:D12 pupate 1 day earlier, but reach a similar weight (Bogus et al., 1987; Kryspin et al., 1974). Similar to these observations, here we did not notice any considerable differences in development of larvae grown under artificial light (L14:D10) vs. in the dark. The effect of illumination on the immune response of G. mellonella remains unexplored and could be of interest to study in the future.

To keep conditions stable, it is important to use the right housing conditions for each stage of the G. mellonella life cycle. Containers in which larvae are kept should be ventilated as feeding and developing larvae produce a lot of heat, increasing the temperature of the colony. When larvae start to pupate, combining multiple jars in one box not only improves ventilation and pupal formation, but also increases genetic diversity. To sustain a healthy G. mellonella colony, it is important to keep the genetic diversity high, as inbreeding depression has detrimental effects in Lepidoptera (Roush, 1986; Higashiura et al., 1999; Saccheri et al., 2005).

It is often mentioned that only cream-colored larvae should be used for experiments as pigmentation is related to disease and stress (Jorjão et al., 2018; Firacative et al., 2020; Pereira & Rossi, 2020). However, G. mellonella naturally varies from creamy white to ash grey (Figure 1) (Dubovskiy et al., 2013; Desai et al., 2019). Melanic morphs of G. mellonella have even been linked to increased fungal resistance (Dubovskiy et al., 2013). Therefore, equal greyish pigmentation of the dorsal side is not necessarily a sign of disease. However, the ventral side of the larvae should be creamy white. Grey markings associated to...
disease are often unevenly dispersed and visible at the ventral side of the larvae (Figure 1A4, B4). Although both white and grey larvae can be used for infection experiments, for standardization purposes it is best to only use larvae of one color. Further investigation is needed to see which type of larvae can be used best for infection experiments.

Unifying rearing protocols is the next step in standardizing propagation methods, which helps to develop G. mellonella as a robust, reliable, and consistent infection model and thus a good alternative invertebrate model. Maintaining an in-house colony of G. mellonella not only increases quality and reproducibility of experiments, it also secures the continuity of these. Our protocol produced high-quality larvae with a short life cycle, increased weight, and higher resistance to infection with C. albicans compared to commercially bred larvae. The low costs and easy to obtain materials makes it convenient to implement this setup in other laboratories. Using the same G. mellonella rearing protocol and the inclusion of a standard fungal reference strain (e.g., C. albicans CBS562 and/or CBS6421) during experimental work will greatly improve inter-laboratory comparisons of virulence studies.

AUTHOR CONTRIBUTIONS
Auke W. de Jong: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). Dennis van Veldhuizen: Methodology (equal); validation (equal); writing – review and editing (equal). Astrid T. Groot: Conceptualization (equal); resources (equal); supervision (equal); writing – review and editing (equal). Ferry Hagen: Conceptualization (equal); funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available via the Data Archiving and Networking Services (DANS-KNAW) at https://doi.org/10.17002/dans-xqv-eztn.

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