The Galleria mellonella-Enteropathogenic Escherichia coli Model System: Characterization of Pathogen Virulence and Insect Immune Responses

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Abstract

The use of Galleria mellonella (Linnaeus) (Lepidoptera: Pyralidae), an economical insect model, for the study of enteropathogenic Escherichia coli (Migula) (EPEC), a diarrheagenic human pathogen, has been demonstrated previously but remains poorly understood. The present study characterizes the Galleria-EPEC system extensively for future studies using this system. We found that EPEC causes disease in G. mellonella larvae when injected intrahemocoelically but not orally. Disease manifests as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, increased pupal duration, and hemocytopenia. Disease symptoms are dose-dependent and can be used as metrics for measuring EPEC virulence in future studies. The type III secretion system was only partially responsible for EPEC virulence in G. mellonella while the majority of the virulence remains unknown in origin. EPEC elicits insect anti-bacterial immune responses including melanization, hemolymph coagulation, nodulation, and phagocytosis. The immune responses were unable to control EPEC replication in the early stage of infection (≤3 h post-injection). EPEC clearance from the hemocoel does not guarantee insect survival. Overall, this study provided insights into EPEC virulence and pathogenesis in G. mellonella and identified areas of future research using this system.

Key words: Galleria mellonella, enteropathogenic Escherichia coli, insect model, virulence, immune response

Escherichia coli is a Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae. The six major pathotypes of diarrheagenic E. coli are enteropathogenic E. coli (EPEC), enteroaggregative E. coli (EAEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC), Shiga toxin-producing E. coli (STEC), and diffusely adherent E. coli (DAEC). EPEC differs from other pathotypes by displaying attaching and effacing (A/E) properties and lacking Shiga toxins. EPEC causes diarrhea in humans, especially in children of developing countries. The only known reservoir hosts of EPEC are humans and is transmitted through the ingestion of contaminated material (fecal-oral). EPEC causes disease in the small intestine of the host and the colonization process is initiated by the attachment of EPEC to enterocytes by bundle-forming pili. The injection of effectors by EPEC into the enterocyte through a type III secretion system (T3SS) results in the destruction (effacement) of the microvilli and the formation of the characteristic actin pedestals on the apical surface of the enterocyte by cytoskeletal rearrangement. Diarrhea is likely caused by a combination of EPEC effectors that inhibit intestinal fluid uptake, alter water and ion transport, and increase intestinal permeability (Croxen et al. 2013).

Currently, the mouse (Mus musculus) is the model host used for in vivo studies of EPEC, either directly or through the use of a surrogate murine enteropathogen Citrobacter rodentium that is similar to EPEC (Dupont et al. 2016, Rhee et al. 2011; Shifflett et al. 2005). Mice are anatomically, physiologically, and genetically similar to humans, making them ideal animal models to study human pathogens and diseases (Vandamme 2015). However, studies using mice are constrained by the high costs of maintenance, low sample sizes, and ethical concerns compared to invertebrate models (Pereira et al. 2018). These constraints can be alleviated by the use of insect models such as the greater wax moth (Galleria mellonella), in which human pathogens (e.g., Candida albicans and Pseudomonas aeruginosa) show similar virulence as murine models (Brennan et al. 2002, Jander and Rahme 2000). G. mellonella is an economically important pest of honey bees worldwide (Kwadha et al. 2017). The larvae feed primarily on honey, pollen, and wax in honey bee hives causing extensive damage to the honey combs and entrap emerging bees with silk in a condition known as galleriass (Williams 1997). In the laboratory, the larvae are used as a model organism to study numerous human pathogens including bacteria, fungi, and protozoans (Aperis et al. 2007, Fedhila et al. 2006, Fuchs et al. 2010, Zoetz et al. 2018).
Jackson et al. 2009, Leuko and Raivio 2012, Miyata et al. 2003, Mukherjee et al. 2010, Mylonakis et al. 2005, Peleg et al. 2009, Seed and Dennis 2008, Tomirotto-Pellissier et al. 2016). The use of *G. mellonella* as an alternative model host to study EPEC was first suggested and demonstrated by Leuko and Raivio (2012). In this study, *G. mellonella* larvae were shown to be susceptible to E2348/69 (serotype O127:H6), the prototypical EPEC strain, in a dose-dependent manner after intrahemocoelic injection. A mutant strain of EPEC without functional T3SS was less virulent than the wild type, indicating that the secreted effectors are important in EPEC virulence in *G. mellonella*. The Cpx envelope stress response, that protects EPEC from envelope protein misfolding, is also implicated in EPEC virulence in *G. mellonella*. The mechanisms of EPEC virulence in *G. mellonella* remain unknown. EPEC was not seen attached to *G. mellonella* tissues and the EPEC mutant lacking bundle-forming pili showed similar virulence as the wild-type, indicating that EPEC attachment to host cells may not be required for virulence in *G. mellonella* (Leuko and Raivio 2012). Nothing is known about the sublethal effects of EPEC to *G. mellonella*, which could be used as metrics for measuring EPEC virulence in addition to mortality. The use of multiple metrics of virulence may help future studies identify virulence factors of EPEC in *G. mellonella* where the use of mortality alone is insufficient. Virulence factors identified using the *Galleria*-EPEC model system could subsequently be examined in the murine model to determine whether such factors are important in EPEC virulence in humans. Typical insect immune responses against bacteria are melanization, hemolymph coagulation, AMP production, phagocytosis, and nodulation (Cytrynska et al. 2016). The immune responses induced by enteropathogenic *Escherichia coli* (EPEC) in *G. mellonella* are not well characterized, which to date only include melanization, nodulation, and the expression of the AMPs cecropin and gloverin (Leuko and Raivio 2012). In addition, almost nothing is known about the events occurring inside the insect post-injection with EPEC, which are important in understanding both EPEC pathogenesis and *G. mellonella* immunity for the establishment of the *Galleria*-EPEC model system.

The present study aims to increase our understanding of the *Galleria*-EPEC model system by the characterization of EPEC virulence and pathogenicity in *G. mellonella* and *G. mellonella* immune responses against EPEC. The definitions of virulence and pathogenicity proposed by Thomas and Elkinton (2004) are used, in which virulence refers to the ability of a pathogen to cause disease in infected hosts whereas pathogenicity includes virulence while also accounting for the infectivity of the pathogen. The nature of EPEC virulence in the hemocoel of *G. mellonella* was determined by monitoring insect mortality, survival time, time to pupation, pupal mass, pupal duration, fecundity, and egg hatch rate after intrahemocoelic injection at various doses of EPEC. The source of EPEC virulence in *G. mellonella* was examined by comparing the intrahemocoelic LD₅₀ of EPEC to the LD₅₀ of an EPEC mutant (ΔescN) with disabled T3SS and to the LD₅₀ of a benign *E. coli* strain (DH5α). If the T3SS is the main or only source of EPEC virulence in *G. mellonella*, then the LD₅₀ of ΔescN would be similar to the LD₅₀ of DH5α. The degree of EPEC virulence in *G. mellonella* was examined by comparing the intrahemocoelic LD₅₀ of EPEC to that of a known entomopathogen (*Providencia rettgeri*) and a benign soil bacterium (*Bacillus clausii*). The pathogenicity of EPEC in *G. mellonella* was examined by comparing the intrahemocoelic LD₅₀ to the per os LD₅₀ of EPEC. The per os injection was conducted since it represents one of the most common portals of entry for bacterial pathogens in insects in nature as well as the portal of entry for EPEC in humans (Croxon et al. 2013, Tanada and Kaya 1993b). The immune responses of *G. mellonella* against EPEC were characterized in vivo by hemolymph examination following intrahemocoelic EPEC injection. The temporal dynamics of circulating hemocytes, melanized particles, nodules, and EPEC replication/clearance during EPEC infection in *G. mellonella* were determined by quantification using hemocytometer and the plate-count method following intrahemocoelic EPEC injection. If EPEC activates humoral and cellular immune responses in *G. mellonella*, then the number of circulating hemocytes should decrease over time while the number of melanized particles and nodules should increase over time. If *G. mellonella* immune responses are effective in controlling the EPEC infection in the hemocoel, then the number of circulating EPEC should also decrease over time.

We found that EPEC-induced disease in *G. mellonella* was dose-dependent and manifested as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. EPEC had moderate virulence in *G. mellonella* relative to an entomopathogenic bacterium and a soil bacterium but had low oral pathogenicity. The T3SS contributed to EPEC virulence in *G. mellonella* but unknown factors were responsible for most of the virulence, opening additional avenues for future research. Hemocytopenia was temporarily induced in *G. mellonella* by EPEC between 3–6 h post-injection but the circulating hemocyte count recovered by 48 h post-injection. The immune responses of *G. mellonella* were unable to control EPEC replication at the LD₅₀ in the early stage of infection (i.e., within 3 h post-injection) but were eventually able to clear EPEC from the hemolymph by 48 h post-injection. The clearance of circulating EPEC corresponded to the appearance of melanized particles and nodules, implicating these insect immune responses in EPEC clearance. Interestingly, EPEC clearance did not guarantee insect survival, likely due to irrecoverable damage from EPEC and the immune responses.

Overall, this study provided insights into EPEC pathogenesis in *G. mellonella* and *G. mellonella* immune responses against EPEC needed for future research and identified life history metrics for the evaluation of EPEC virulence in *G. mellonella*.

### Materials and Methods

#### Insect Rearing

*G. mellonella* larvae were purchased from Recorp Inc. (Georgetown, Ontario, Canada) and used to establish a laboratory colony. Insects were reared in 20 oz Atlas mason jars maintained in a Percival I-41VL incubator at 30°C and 30% RH in total darkness (0L:24D) and fed ad libitum on an artificial diet (Supp Appendix 1 [online only]). Last instar larvae, approximately 300 mg in mass, (Mettler College150 digital precision balance) were used for all experiments described in this study.

#### Bacteria Strains, Culturing, and Quantification

Wild type EPEC (E2348/69 serotype O127:H6) and an EPEC mutant ΔescN were obtained from T. L. Raivio (University of Alberta). The ΔescN mutant of EPEC lacks the ability to secrete effectors using the T3SS due to the loss of the EscN protein that functions as an ATPase for the T3SS (Andrade et al. 2007). EPEC was transformed with the plasmid pXG-1, enabling the constitutive expression of green fluorescent protein (GFP) for in situ visualization by fluorescence microscopy and chloramphenicol resistance for isolation by selective media (Urban and Vogel 2007). *E. coli* strain DH5α was obtained from P. D. Batista (University of Northern British Columbia). *P. rettgeri* was isolated from *G. mellonella* killed by *Steinernema carpocapsae* which were obtained from...
Survival Score = \frac{\text{Survival Time}}{20}

which were used as proxies for survival time in the analysis to avoid heteroscedasticity. Survival time of insects that died as pupae could not be determined and was not included in the survival time analysis. Time to pupation was recorded as the number of days post-injection until pupation. Larvae were considered dead when no movement was observed after tactile stimulation.

Sublethal Effects of EPEC on G. mellonella

Known sublethal doses of EPEC were injected intrahemocoelically into 45 G. mellonella larvae (0 CFU, 1.4 × 10^2 CFU, 1.4 × 10^3 CFU, 2.1 × 10^3 CFU, and 4.2 × 10^3 CFU) (Supp Appendix 3.2 [online only]). Time to pupation, pupal mass, adult eclosion, fecundity, and egg hatch rate were recorded. Pupae were carefully extracted from cocoons using micro scissors and fine tip forceps following sclerotization (color change from light yellow to dark brown) of the pupal cuticle. Pupae damaged during the extraction were removed from the experiment. After pupal mass was measured on a digital precision balance (Mettler College150) and recorded within 24 h post-injection to minimize the effects of water loss, each pupa was sexed and placed into a 1 oz Solo plastic cup. Pupal duration was calculated as the time difference between pupation and eclosion (adult emergence) post-injection. Each adult female, within 24 h after eclosion, was transferred into a new 1 oz Solo plastic cup with an untreated adult male and incubated at 30°C. A thin film of artificial diet was smeared onto the inner surface of each cup to facilitate oviposition. The number of eggs in each cup was recorded after the death of the female. Twenty eggs were randomly collected from each cup and placed into 2 ml microfuge tubes at 30°C and hatching was monitored.

Route of Infection and EPEC Pathogenicity

Four doses of EPEC (0 CFU, 2.2 × 10^6 CFU, 2.5 × 10^6 CFU, 1.1 × 10^7 CFU, and 2.5 × 10^7 CFU) were injected per os into the midgut of 70 G. mellonella larvae (Supp Appendix 3.3 [online only]). Insect mortality, survival time, time to pupation, pupal mass, fecundity, and egg hatch rate were recorded as previously described.

EPEC Virulence Compared to Other Bacteria

Various doses of E. coli (ΔescN: 0 CFU, 4.5 × 10^5 CFU, 4.5 × 10^6 CFU, 4.9 × 10^5 CFU, 7.4 × 10^4 CFU, 9.7 × 10^4 CFU, 1.1 × 10^4 CFU, 1.5 × 10^4 CFU, 2.9 × 10^5 CFU, 4.5 × 10^5 CFU, and 9.0 × 10^6 CFU; n = 110) (DH5α: 0 CFU, 2.0 × 10^1 CFU, 2.0 × 10^2 CFU, 2.0 × 10^3 CFU, 2.0 × 10^4 CFU, 1.9 × 10^5 CFU, 7.8 × 10^6 CFU, and 4.1 × 10^7 CFU; n = 85), P. rettgeri (0 CFU, 1.8 × 10^2 CFU, 1.8 × 10^3 CFU, 1.8 × 10^4 CFU, 1.8 × 10^5 CFU, 2.6 × 10^6 CFU; n = 50), and B. clausii (0 CFU, 3.5 × 10^2 CFU, 3.5 × 10^3 CFU, and 4.6 × 10^6 CFU; n = 55) were injected intrahemocoelically into G. mellonella larvae (Supp Appendix 3.4 [online only]). Insect mortality was recorded.

Characterization of G. mellonella Immune Responses

The LD_{50} of EPEC (approximately 1.5 × 10^4 CFU) was injected intrahemocoelically into G. mellonella larvae as previously described. Larvae injected with Ringer’s were used as control. Larvae were surface-sterilized (by immersion: 30 s in 70% ethanol → 10 s in sterile water → 60 s in 10% bleach → 10 s in sterile water)
immediately before hemolymph collection. Hemolymph was collected aseptically at 24 h post-injection from 42 insects (28 injected with EPEC, 14 injected with Ringer’s) by micropipette after creating a small incision at the base of the right anteriormost proleg (or, if not available, the left second-anteriormost proleg) with micro scissors and the application of gentle pressure to the insect until a droplet of hemolymph appears. Hemolymph (DNA) was stained with Hoechst 33342 (10 µg/mL, Riedel-de Haën) without fixing to minimize handling, since the stain is live cell-permeable. Hemolymph collection and staining were performed aseptically in a biological safety cabinet (Model 1106, Forma).

Quantification of Circulating Hemocytes, Nodules, Melanized Particles, and EPEC

_G. mellonella_ larvae were injected intrahemocoelically with EPEC (1.5 x 10^4 CFU, n = 60) and heat-killed EPEC (HK-EPEC, 1.5 x 10^4 CFU before killing, n = 60). Insects injected with Ringer’s (n = 60) and sham-injected insects (n = 60) were used as controls. HK-EPEC was prepared by heating the bacteria to 65°C for 30 min in a heating block (Isotemp Model 145, Fisher). Cell integrity was verified by DIC microscopy and complete killing was confirmed by plate-count. Sham injection was performed identically to standard intrahemocoelic injections except with no inoculum injected (i.e., wounding by needle insertion). Hemolymph was collected aseptically from 8 insects of each treatment, randomly without replacement, at 1 h, 3 h, 6 h, 24 h, and 48 h post-injection. All insects were alive at the time of hemolymph collection. Hemolymph was immediately diluted ten-fold in an anticoagulant antimelanization solution (Supp Appendix 2.2 [online only]) post-collection to prevent ex vivo hemolymph coagulation and melanization. Hemolymph samples from each insect were loaded onto an improved Neubauer hemocytometer to quantify circulating hemocytes, nodules, and melanized particles by brightfield and phase contrast microscopy using an Olympus CX41 microscope at 400x magnification. The proportion of hemocytes in microaggregations were calculated for each sample:

\[
\text{Proportion of hemocytes in microaggregations} = \frac{\text{Number of hemocytes in microaggregations}}{\text{Total number of hemocytes}}
\]

as proxies for hemocyte adhesiveness and activation. Only mature nodules with multiple layers of hemocytes surrounding a melanized mass of bacteria were counted (Ratcliffe and Gagen 1976). The same hemolymph samples were also used to quantify circulating bacteria by plate-count. The remaining insects (20 of each treatment) were left undisturbed to monitor development and mortality. Hemolymph was collected aseptically from dead insects within 24 h post-mortem to quantify EPEC by plate-count on LB agar with 1 mg/ml chloramphenicol to control for potential bacterial contamination from loss of gut integrity. Insect rearing condition, bacteria culture condition, injection protocol, and incubation condition post-injection were as described previously.

Statistical Analyses

Statistical analyses in this study were conducted using R (R Core Team 2019). GLMs were constructed to examine: (1) relationships between bacteria dose and insect survival time, time to pupation, pupal mass, pupal duration, fecundity, and egg hatch rate; (2) differences in hemocyte count and hemocyte microaggregation between different treatments, time points, and their interactions; (3) differences in melanized particle and bacteria counts between different time points in insects injected with EPEC; and (4) differences in the number of days to pupation post-injection between different treatments. The minimum adequate models were obtained by stepwise deletion of non-significant factors and interactions when applicable. Model comparisons were conducted using F tests or χ² tests. Tukey contrasts (pairwise comparisons) were used to determine where significant differences occurred post hoc to GLMs. The median lethal dose (LD₅₀) was defined in the context of this study as the number of bacteria injected that would kill 50% of the insects by the end of the experiment. The LD₅₀ values of each bacteria species and strain were determined by binomial or quasi-binomial GLMs using the probit link function.

Results

EPEC and _G. mellonella_ Mortality

The intrahemocoelic LD₅₀ of EPEC in _G. mellonella_ larvae is 1.58 x 10⁴ ± 1.26 x 10³ CFU (±95% CI) (Fig. 1). No mortality was observed in insects injected with ≤ 5 x 10³ CFU. EPEC dose was a significant predictor of insect mortality (Binomial GLM, deviance = 301, df = 1 and 347, P < 0.0001), melanization (Quasi-binomial GLM, F = 821, df = 1 and 347, P < 0.0001), survival score (Quasi-binomial GLM, F = 670, df = 1 and 342, P < 0.0001), and time to pupation (Quasi-Poisson GLM, F = 188, df = 1 and 219, P < 0.0001). Increase in EPEC dose was associated with increase in mortality (β = 1.79 x 10⁻⁴, P < 0.0001), increase in melanization (β = 4.16 x 10⁻⁴, P < 0.0001), decrease in survival score (β = −2.51 x 10⁻⁴, P < 0.0001), and increase in time to pupation (β = 3.13 x 10⁻⁵, P < 0.0001) (Figs. 1 and 2). Melanization was a significant predictor of insect mortality (Binomial GLM, deviance = 339, df = 1 and 347, P < 0.0001), survival score (Quasi-binomial GLM, F = 551, df = 1 and 342, P < 0.0001), and time to pupation (Poisson GLM, deviance = 12.8, df = 1 and 219, P = 0.0004). No signs of melanization were observed in control larvae injected with Ringer’s. Larvae that displayed moderate to severe melanization (Fig. 3c and d) showed significantly higher mortality (z = 13.6, P < 0.0001), lower survival score (t = −9.44, P < 0.0001), and longer time to pupation (z = 3.77, P = 0.0002) compared to insects that showed slight to no melanization (Fig. 3a and b) (Fig. 5). Insect mortality occurred at either the larval stage (96.1%) or the pupal stage (3.9%). EPEC dose was not a significant predictor of whether insect mortality occurs during the larval stage or the pupal stage (Binomial GLM, deviance = 2.2, df = 1 and 127, P = 0.14). Insects that died within 24 h post-injection showed massive EPEC presence in the hemolymph (personal observation). Moribund larvae cease to feed (anorexia),
show minimal movement (lethargy), gradually shrink over time (brachytosis), and can remain alive for up to 20 d post-injection before death eventually occurs. Black feces (frass) and diarrhea were observed from moderately to severely melanized larvae by 24 h post-injection (Fig. 4a). Larvae injected with Ringer’s produced normal brown frass without any diarrhea (Fig. 4b). Careful dissection of dead pupae (usually shriveled and slightly deformed) revealed fully developed pharate adults under the pupal cuticle. Adults that successfully eclosed from pupae appear normal in all treatments.

Sublethal Effects of EPEC on G. mellonella

EPEC dose was a significant predictor of time to pupation (Quasi-Poisson GLM, $F = 17.8$, df = 1 and 37, $P = 0.0002$). Increase in EPEC dose was associated with increase in time to pupation in G. mellonella regardless of sex ($\beta = 5.20 \times 10^{-3}$, $P = 0.0001$) (Fig. 6a). EPEC dose and insect sex were significant predictors of pupal mass (Gaussian GLM; $F = 5.99$, df = 1 and 36, $P = 0.02$; $F = 27.8$, df = 1 and 36, $P < 0.0001$; respectively). EPEC dose did not affect the sexes differently ($F = 0.119$, df = 1 and 35, $P = 0.73$). Pupal mass decreased as EPEC dose increased ($\beta = -8.24 \times 10^{-3}$, $P = 0.02$), with female pupae being 55.1 mg more massive on average than male pupae ($t = 5.27$, $P < 0.0001$) (Fig. 6b). EPEC dose and insect sex were significant predictors of pupal duration (Quasi-Poisson GLM, $F = 32.0$, df = 1 and 36, $P = 0.0001$; $F = 18.4$, df = 1 and 36, $P = 0.0001$; respectively). EPEC dose did not affect the sexes differently ($F = 0.119$, df = 1 and 35, $P = 0.73$). Pupal duration increased as EPEC dose increased ($\beta = 6.34 \times 10^{-5}$, $P < 0.0001$), with female pupae taking 1.15 d longer on average than male pupae to complete metamorphosis ($t = 4.23$, $P = 0.0002$) (Fig. 6c). EPEC dose was not a significant predictor of fecundity (Gaussian GLM, $F = 0.459$, df = 1 and 28, $P = 0.50$) or egg hatch rate (Quasi-binomial GLM, $F = 1.30 \times 10^{-3}$, df = 1 and 36, $P = 0.97$). Pupal mass was not a significant predictor of fecundity (Gaussian GLM, $F = 0.433$, df = 1 and 28, $P = 0.52$) or egg hatch rate (Quasi-binomial GLM, $F = 1.97$, df = 1 and 23, $P = 0.17$).

Route of Infection and EPEC Pathogenicity

The per os LD$_{50}$ of EPEC in G. mellonella larvae was greater than $2.50 \times 10^7$ CFU (5% mortality at this dose, $n = 20$), which was more than 3 orders of magnitude higher than the intrahemocoelic LD$_{50}$ ($1.58 \times 10^4 \pm 1.26 \times 10^3$ CFU) (Table 1). EPEC dose was not a significant predictor of insect survival score (Quasibinomial GLM, $F = 1.38$, df = 1 and 68, $P = 0.24$), time to pupation (Poisson GLM, deviance = 0.546, df = 1 and 66, $P = 0.46$), pupal mass (Gaussian GLM, $F = 0.226$, df = 1 and 63, $P = 0.64$), pupal duration (Quasi-Poisson GLM, $F = 9.88 \times 10^{-5}$, df = 1 and 63, $P = 0.75$), and fecundity (Gaussian GLM, $F = 0.677$, df = 1 and 45, $P = 0.42$). Female pupae were 37.7 mg larger than male pupae on average ($t = 3.83$, $P = 0.0003$) and took 1.26 d on average longer to complete metamorphosis ($t = 4.28$, $P < 0.0001$). EPEC dose was a significant predictor of egg hatch rate (Quasi-binomial GLM, $F = 5.57$, df = 1 and 28, $P = 0.00003$) and took 1.26 d on average longer to complete metamorphosis ($t = 4.28$, $P < 0.0001$). EPEC dose was a significant predictor of egg hatch rate (Quasi-binomial GLM, $F = 5.57$, df = 1 and 28, $P = 0.00003$) and took 1.26 d on average longer to complete metamorphosis ($t = 4.28$, $P < 0.0001$). EPEC dose was a significant predictor of egg hatch rate (Quasi-binomial GLM, $F = 5.57$, df = 1 and 28, $P = 0.00003$) and took 1.26 d on average longer to complete metamorphosis ($t = 4.28$, $P < 0.0001$).
41, \( P = 0.02 \)). EPEC dose was negatively associated with egg hatch rate (\( \beta = -6.94 \times 10^{-8}, P = 0.02 \)) (Fig. 7). Pupal mass was not a significant predictor of fecundity (Gaussian GLM, \( F = 1.43, \text{df} = 1 \) and 45, \( P = 0.24 \)) or egg hatch rate (Quasi-binomial GLM, \( F = 1.93, \text{df} = 1 \) and 41, \( P = 0.17 \)). No abnormal frass or diarrhea were observed post-injection.

**EPEC Virulence Compared to Other Bacteria**

The LD\(_{50}\) of EPEC was approximately 3 times lower than the LD\(_{50}\) of the \( \Delta \)escN mutant in *G. mellonella* and more than 3 orders of magnitude lower than the LD\(_{50}\) of the DH5\( \alpha \) strain (Table 1). The LD\(_{50}\) of EPEC was approximately 9 times higher than the LD\(_{50}\) of *P. rettgeri* and more than 2 orders of magnitude lower than the LD\(_{50}\) of *B. clausii* (Table 1).

**Characterization of *G. mellonella* Immune Responses**

Melanization, hemolymph coagulation, nodulation, and phagocytosis were observed in the hemolymph of *G. mellonella* larvae at 24 h post-injection with EPEC. The immune responses were absent in the control larvae injected with Ringer’s (Fig. 8a—c). The following were observed in the hemolymph of larvae injected with EPEC: (1) melanized particles (in the hemolymph of 89% of the larvae) free-floating in the hemolymph, internalized in hemocytes, embedded in coagula, and in the center of nodules (Fig. 8d—g); (2) hemolymph coagulation around melanized particles (Fig. 8f); (3) nodules containing melanized particles (Fig. 8g); and (4) hemocytes containing EPEC, indicative of phagocytosis (Fig. 8h—i). No discernible differences in hemolymph low molecular weight protein profile representing antimicrobial peptides (AMPs) were detected between *G. mellonella* injected with EPEC compared to the control (SDS–PAGE and silver staining of hemolymph protein extracted at 24 h post-injection, data not shown).

**Quantification of Circulating Hemocytes, Nodules, Melanized Particles, and EPEC**

Statistically significant differences in circulating hemocyte count between treatments (Quasi-Poisson GLM, \( F = 14.8, \text{df} = 3 \) and 156,
were found (Fig. 9). No differences were detected in hemocyte count between treatments over time (i.e., no significant interaction between treatment and time; $F = 1.32$, df = 12 and 140, $P = 0.21$). Larvae injected with EPEC showed a significantly reduced number of circulating hemocytes compared to larvae injected with either Ringer’s, HK-EPEC, or sham-injected larvae (Tukey contrasts, $P \leq 0.0001$ in all comparisons). No significant differences in circulating hemocyte count were detected between larvae injected with Ringer’s, HK-EPEC, and sham-injected larvae ($P \geq 0.73$ in all comparisons). A significant difference in circulating hemocyte count was detected between 3 h and 6 h post-injection overall (Tukey contrasts, $P = 0.05$). No statistically significant differences were found in hemocyte microaggregation (proportion of hemocytes in microaggregations) between treatments (Quasi-binomial GLM, $F = 2.13$, df = 3 and 156, $P = 0.10$), between time points ($F = 0.920$, df = 4 and 152, $P = 0.45$), and between treatments over time ($F = 0.349$, df = 12 and 140, $P = 0.98$). Nodules were not observed in the hemolymph of larvae injected with Ringer’s, HK-EPEC, and sham-injected larvae at any time point post-injection. Statistically significant differences in nodule count between time points were found in the hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 4.25$, df = 4 and 35, $P = 0.007$). Nodules began to appear in the hemolymph at 6 h post-injection and were absent from the hemolymph by 48 h post-injection (Fig. 10). No melanized particles were found in the hemolymph of larvae injected with Ringer’s and sham-injected larvae. Melanized particles (100 µl hemolymph at 6 h post-injection) were found in 1 larva out of the 40 (2.5%) injected with HK-EPEC whereas 19 larvae out of the 40 (47.5%) injected with EPEC were found with melanized particles in the hemolymph. Statistically significant differences in the number of melanized particles between time points were found in the hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 11.5$, df = 4 and 35, $P < 0.0001$). Melanized particles began to appear in the hemolymph at 6 h post-injection and were also found at 24 h and 48 h post-injection (Fig. 10). Melanization occurred at the injection wound site in all insects. Bacteria were not observed in the hemolymph of larvae injected with Ringer’s, HK-EPEC, and sham-injected larvae at any time point post-injection, indicating the absence of culturable bacteria in the hemolymph of G. mellonella. Statistically significant differences in bacteria count between time points were found in the hemolymph of larvae injected with EPEC (Quasi-Poisson GLM, $F = 14.5$, df = 4 and 35, $P < 0.0001$). The number of bacteria increased from 1 h to 3 h post-injection (Tukey contrasts, $P < 0.0001$), decreased from 3 h to 6 h post-injection ($P = 0.002$), did not change between 6 h to 24 h post-injection ($P = 0.99$) and were cleared from the hemolymph by 48 h post-injection ($P = 1.0$) (Fig. 10). The reduction in bacteria count was concurrent with the appearance of melanized particles and

![Fig. 6. Time to pupation (a), pupal mass (b), and pupal duration (c) of G. mellonella larvae injected intrahemocoecically with sublethal doses of EPEC (n = 45). (a) Time to pupation post-injection increased as EPEC dose increased ($\beta = 5.20 \times 10^{-6}$, $P = 0.0001$). (b) Pupal mass decreased as EPEC dose increased ($\beta = -8.24 \times 10^{-7}$, $P = 0.02$), with female pupae being 55.1 mg larger on average than male pupae ($t = 5.27$, $P < 0.0001$). (c) Pupal duration increased as EPEC dose increased ($\beta = 6.34 \times 10^{-6}$, $P < 0.0001$), with female pupae taking 1.15 d longer on average than male pupae to complete metamorphosis ($t = 4.23$, $P = 0.0002$).](image)

![Fig. 7. Egg hatch rate of G. mellonella larvae injected per os with various doses of EPEC (n = 70). Increase in EPEC dose was associated with a marginal decrease in egg hatch rate ($\beta = -6.94 \times 10^{-8}$, $P = 0.02$).](image)

### Table 1. The LD$_{50}$ of different species and strains of bacteria in G. Mellonella

| Bacteria           | Route of injection | LD$_{50}$ ± 95% CI (CFU) | n  |
|--------------------|--------------------|---------------------------|----|
| E. coli (EPEC)     | Intrahemocoelic   | $1.58 \times 10^{4}$ ± $1.26 \times 10^{3}$ | 349 |
| E. coli (ΔescN)    | Intrahemocoelic   | $4.72 \times 10^{4}$ ± $5.41 \times 10^{3}$ | 110 |
| E. coli (DH5α)     | Intrahemocoelic   | $4.10 \times 10^{4}$ ± $1.53 \times 10^{4}$ | 85  |
| P. rettgeri        | Intrahemocoelic   | $1.78 \times 10^{3}$ ± $7.70 \times 10^{2}$ | 50  |
| B. clausii         | Intrahemocoelic   | $3.73 \times 10^{6}$ ± $1.08 \times 10^{6}$ | 55  |
| E. coli (EPEC)     | Per os             | $> 2.50 \times 10^{7}$     | 70  |
Fig. 8. Typical immune responses observed in the hemolymph of *G. mellonella* larvae at 24 h after intrahemocoelic injection with $1.5 \times 10^4$ EPEC. (a—c) Hemocytes from a control larva injected with Ringer’s displaying: (a) typical hemocyte morphology, (b) typical hemocyte nuclei (blue fluorescence by Hoechst 33342 staining), and (c) absence of green fluorescence. (d) Extracellular melanized particle. (e) Melanized particle internalized by a hemocyte. (f) Hemolymph coagulation around melanized particles. (g) Nodule containing numerous melanized particles. (h—j) Microaggregation of hemocytes (h) with internal green fluorescence (j, arrowhead) at the same focal plane as hemocyte nuclei (i) indicating phagocytosis of EPEC.

Fig. 9. The average number of circulating hemocytes (±SE) per µl hemolymph at different time points in *G. mellonella* larvae injected intrahemocoelically with Ringer’s, EPEC, HK-EPEC, or sham-injected (n = 160). Larvae injected with EPEC showed reduced hemocyte count compared to the other treatments ($F = 14.8, P < 0.0001$), most prominently at 6 h post-injection (74% reduction relative to Ringer’s). Within the EPEC treatment, hemocyte count reduced by 66% between 3 h and 6 h post-injection but recovered between 24 h and 48 h post-injection. The hemocyte count for the other treatments remained relatively constant across all time points.

Fig. 10. The average number of circulating EPEC, melanized particles, and nodules (±SE) per µl hemolymph at different time points in *G. mellonella* larvae injected intrahemocoelically with EPEC (n = 40). The reduction in the number of circulating EPEC is followed by the appearances of melanized particles and nodules in the hemolymph.
nodules (Fig. 10). All insects left undisturbed from sham, Ringer’s, and HK-EPEC treatments survived and eclosed as adults whereas 60% mortality (12 out of 20) was observed from the larvae injected with EPEC. EPEC was found in the hemolymph of only 1 larva (died at 48 h post-injection with too many CFUs to count) out of the 12 that died. Statistically significant differences in the number of days to pupation between larvae of different treatments were detected (Quasi-Poisson GLM, $F = 32.8, df = 3$ and 64, $P < 0.0001$). Pupation in larvae injected with EPEC was delayed an average of 4 d compared to larvae injected with Ringer’s, HK-EPEC, or sham-injected larvae (Tukey contrasts, $P < 0.0001$ in all comparisons) (Fig. 11). No statistically significant differences in the number of days to pupation were detected between larvae injected with Ringer’s, HK-EPEC, and sham-injected larvae ($P \geq 0.72$ in all comparisons) (Fig. 11).

### Discussion

**EPEC and *G. mellonella* Mortality**

The dose-dependent increase in insect mortality (Fig. 1) and decrease in survival time (Fig. 2a) following EPEC injection demonstrate that EPEC causes disease in *G. mellonella* larvae when injected into the hemocoel. Ringer’s alone did not kill any insects (Fig. 1), indicating that injection trauma and blank inoculum do not cause insect mortality. The LD$_{50}$ ($1.58 \times 10^4$ CFU) of EPEC in *G. mellonella* larvae in this experiment at day 20 post-injection versus the LD$_{50}$ ($2.57 \times 10^3$ CFU) at 48 h post-injection determined by Leuko and Raivio (2012) is likely due to the difference in the time of observation and differences in the physiological state of the larvae used in these experiments. Leuko and Raivio (2012) obtained the larvae directly from Recorp Inc., stored them at 4°C without diet for ≤ 7 d pre-injection, and incubated them without diet post-injection. Cold shock (at 12°C or 4°C) of *G. mellonella* larvae pre-injection is known to increase resistance to intrahemocoelically injected *Bacillus thuringiensis* and *C. albicans* by increasing both AMP expression and the number of circulating hemocytes (Mowlds and Kavanagh 2008, Wodzi et al. 2014). Starvation, however, may reduce *G. mellonella* resistance to *C. albicans* by reducing AMP expression and the number of circulating hemocytes (Banville et al. 2012). Though it is currently unknown how *G. mellonella* would respond to the combination of cold shock and starvation when faced with an immune challenge, the results obtained under such conditions would not be comparable to those obtained under the optimal conditions used in this study. The short observation time of 48 h used by Leuko and Raivio (2012) to determine the LD$_{50}$ would significantly underestimate true insect mortality by overlooking mortality that occurs at later times. Therefore, it is important to establish and follow a standardized protocol for the rearing and handling of *G. mellonella* as a host for the evaluation of pathogens with the ability to compare results obtained from different laboratories. Recent reviews have identified and discussed this issue in more detail (Champion et al. 2018, Cook and McArthur 2013, Tsai et al. 2016).

In this study, diseased larvae showed melanization (Figs. 2c and 3), reduced survival time (Fig. 2a), and delayed pupation (Fig. 2b). Symptoms of moribund larvae included anorexia, lethargy, brachyptosis, abnormal frass production, and diarrhea. The signs and symptoms of the disease are likely due to a combination of EPEC replication and effector secretion in the hemolymph (septicaemia) and collateral damage by the insect immune responses (immunopathology). The mechanism of EPEC pathogenesis in *G. mellonella* is currently unknown but previous research suggested that EPEC virulence in the hemolymph is reduced by the inactivation of the T3SS and both inactivation and constitutive activation of the Cpx envelope stress response (Leuko and Raivio 2012). In this study, melanization (Fig. 3) was the first visible sign of an immune response against EPEC post-injection, indicating the activation of the PPO cascade. Melanization first occurred at the dorsal vessel, around which melamin can be seen through the cuticle (Fig. 3b and c). This was likely the result of melanized particle accumulation around the ostia (valves) of the dorsal vessel by hemolymph movement and subsequent phagocytosis by sessile perisomial hemocytes (Sigle and Hillyer 2016). The severity of melanization was dose-dependent (Fig. 2c), indicating increasing activation of the PPO cascade with increasing EPEC presence in the hemocoel. Larval melanization could be used as a visual indicator for assessing EPEC virulence, since the degree of melanization was positively associated with mortality, negatively associated with survival time, and positively associated with time to pupation. Scoring of larval melanization, motor activity, cocoon formation, and insect survival have been used previously to construct a health index for *G. mellonella* to assess the virulence of group A Streptococcus (Loh et al. 2013). A similar protocol could be used for EPEC and other pathogens in future studies. EPEC doses ≤ 5.0 × 10$^3$ CFU failed to kill any insects (Fig. 1), indicating that *G. mellonella* immune responses were able to effectively control EPEC at lower doses. Activation of the PPO cascade produces ROS and cytotoxic quinones that damage the Malpighian tubules in *Tenebrio molitor* (Sadd and Siva-Jothy 2006). Malpighian tubules are responsible for nitrogenous waste excretion and osmoregulation in insects, functionally analogous to the vertebrate kidney. Damage to the Malpighian tubules and/or the rectal complex (i.e., sites of water reabsorption) could potentially lead to excess water excretion, resulting in diarrhea that were observed in moribund insects (Fig. 4). Oxidative stress was known to increase mortality and development time in *G. mellonella* (Hyrl et al. 2007). The dose-dependent increase in time to pupation (Fig. 2b) could also be attributed, in part, to the immunopathology of melanization and indicate the presence of sublethal effects of EPEC on *G. mellonella* life history traits. Pupal mortality was not EPEC dose-dependent overall, but no pupal mortality was observed in control insects that were not injected with EPEC, implicating EPEC in pupal mortality. The proximate cause of pupal mortality could be reduced resistance to desiccation, since pharate adults can have difficulties eclosing from desiccated pupae (Tanada and Kaya 1993a). Alternatively, it is possible that eclosion behaviors failed to initiate altogether, which could indicate the disruption of the insect endocrine system (e.g., insufficient levels of...
EPEC dose (when injected intrahemocoelically) or pupal mass, on metamorphosis. Injection of latex beads, which will be encapsulated and melanized in capsules, and coagula, resulting in increased pupal duration. The injection increased larval development time, decreased pupal mass, and induced temporary paralysis in *Pseudoplatysia includens* and *Pseudaletia separata* (Strand et al. 2000). In *M. sexta* challenged with bacteria, a PSP precursor pro-PSP is expressed in the fat body and released into the hemolymph where cleavage by a protease produces the active PSP (Elefterianos et al. 2009). In *P. separata*, GBP inhibits juvenile hormone (JH) esterase activity in the hemolymph which delays larval development by reducing larval feeding and weight gain (Aizawa et al. 2001; Hayakawa 1990, 1991). It is likely that PSP and GBP share similar mechanisms of reducing larval growth and delaying development due to the structural and functional similarities between PSP and GBP (Strand et al. 2000). The lysis of oenocytoids is also induced by GBP, releasing stored PPO for melanization (Matsumoto et al. 2003). As mentioned previously, the oxidative stress of melanization may also contribute to the developmental delay in *G. mellonella* (Hyls et al. 2007). An unidentified plasmatocyte depletor factor, likely an ENF cytokine, was found to be released from *G. mellonella* hemocytes following bacterial challenge in a dose-dependent manner (Chain and Anderson 1983). Future qPCR and RNAi experiments could determine the presence and roles of ENF cytokines in *G. mellonella* immunity. Cessation of feeding observed in moribund *G. mellonella* larvae injected with EPEC in the previous experiment indicates the possibility of a dose-dependent reduction in the feeding of diseased larvae, though future experiments quantifying diet consumption is required to confirm this. Illness-induced anorexia is a common behavioral response to infection in insects (Adam et al. 2007). It was hypothesized to enhance immune responses in diseased insects since reduced lipid intake decreases hemolymph lipid concentration, which increases available apolipophorin III for pathogen recognition (Adam et al. 2010). In general, these results were consistent with a recent study, in which the recovery from *Serratia marcescens* infection increased larval development time, decreased pupal mass, and decreased adult eclosion rate in *Spodoptera frugiperda* (Zhang et al. 2018). Thus, growth inhibition may be a common sublethal effect of bacterial infections in lepidopteran insects.

Complete metamorphosis in insects is a complex process involving extensive histolysis of larval tissue, histogenesis of adult tissue, and larval tissue remodeling into adult tissue (Rolf et al. 2019). Melanin formed during melanization was not removed from the hemocoeol post-infection and remains for the lifetime of the insect (Hillyer 2016). Metamorphosis may be obstructed by the remnants of the immune response such as the presence of melanized nodules, capsules, and coagula, resulting in increased pupal duration. The injection of latex beads, which will be encapsulated and melanized in the hemocoeol, could determine the effects of physical obstructions on metamorphosis.

In *G. mellonella*, fecundity and egg hatch rate are independent of EPEC dose (when injected intrahemocoelically) or pupal mass, indicating that resource allocation into reproduction takes higher priority than growth to maintain fitness. The results were surprising since positive correlations between the size of the female insect and fecundity has been the norm in insects (Honěk 1993). Reduction in fecundity and egg hatch rate may manifest at higher doses of EPEC when resource allocation and ad libitum feeding can no longer compensate for it, which can be examined in future experiments with no artificial diet available to the larvae post-injection. Egg size was not measured in this experiment. It is also possible that the egg size was reduced while fecundity and hatch rate were maintained, though no such differences were noticed visually. The effect of EPEC on *G. mellonella* adult longevity was not examined in this experiment. It is possible that oxidative damage from melanization as a response to EPEC infection could result in reduced adult longevity.

EPEC does not affect the sexes differently. Males completed metamorphosis faster than females on average (protandry) (Fig. 6c). Protandry is common in holometabolous insects and was hypothesized to increase male fitness by increasing potential mating opportunities (Morby and Ydenberg 2001). Female pupae were larger than male pupae on average (Fig. 6b). This female-biased sexual size dimorphism is known in *G. mellonella* (Kwadha et al. 2017).

### Route of Infection and EPEC Pathogenicity

The K-12 strain of *E. coli* could invade the hemocoeol of *G. mellonella* from the gut, though the mechanism of the invasion is unknown (Freitak et al. 2014). A previous experiment determined that EPEC could also invade the hemocoeol of *G. mellonella* larvae from the gut (translocation) without compromising gut integrity (unpublished data). However, the translocation of EPEC was not efficient since the per os LD$_{50}$ was greater than 10$^8$ times higher than the intrahemocoelic LD$_{50}$ (Table 1). The foregut and hindgut possess a cuticular layer, a substantial barrier to bacterial penetration. The midgut, however, is not as protected by having a more porous peritrophic membrane (PM) which separates the gut lumen from the hemocoeol by just a single layer of columnar epithelial cells attached to a basal lamina, making the midgut a likely route of EPEC entry into the hemocoeol (Tanada and Kaya 1993c). The PM and the presence of gut microbiota could potentially hinder EPEC translocation, while the release of AMPs and ROS into the lumen could limit EPEC growth in the midgut (K. Wu et al. 2016b). The pore diameter of *G. mellonella* PM has not been determined to our knowledge but likely falls between 2 and 36 nm, which are too small for bacteria such as *E. coli* (0.25–1.0 µm in diameter) to pass through unassisted (Lehane 1997). EPEC may be able to secrete a chitinase (e.g., ChiA found in *E. coli* K-12 strain) that can potentially damage the PM and allow EPEC access to the midgut epithelium (Francetic et al. 2000). It is possible, but highly unlikely, that the injection process damaged the PM or the midgut epithelium allowing EPEC entry into the hemocoeol despite the blunt needle tip and the care taken to avoid such damage. The mechanism of EPEC translocation remains unknown and requires ultrastructural and histological examination of the PM and the midgut epithelium to determine. The production of normal frass indicates that EPEC did not disrupt gut function when injected per os, but a histological examination of the midgut tissue is required to determine any pathological effect of EPEC on the gut epithelium. Insect survival time was not affected by EPEC. Insect growth and development (i.e., time to pupation, pupal mass, and pupal duration) were not affected by EPEC when introduced per os, in contrast to the previous experiment in which insect growth and development were stunted after intrahemocoelic injection.
of EPEC, indicate that EPEC is not pathogenic to *G. mellonella* by ingestion. However, survival time was only monitored for 20 d post-injection and did not account for adult longevity, which may be affected by EPEC. Taken together, the results indicate that EPEC must enter the hemocoel to become virulent but could not do so efficiently, which is typical for entomopathogenic members of Enterobacteriaceae in insects (Tanada and Kaya 1993d). It is also possible that the gut transit time of *G. mellonella* larvae was too short for EPEC to invade the hemocoel or cause significant pathology in the gut. The examination of frass produced post-injection could help determine the fate of EPEC post-injection. Unexpectedly, egg hatch rate was marginally reduced with increasing EPEC dose injected per os (Fig. 7) whereas egg hatch rate was unaffected when EPEC was injected intrahemocoelically in the sublethal experiment discussed above. The cause of the reduced egg hatch rate is unknown and requires histological examination to determine. How reduced hatch rate is linked to EPEC presence in the gut but not when EPEC was injected into the hemocoel is unknown and may involve gut-specific immune signaling and activation of transgenerational immune priming, which may be costly to eggs (Tetreau et al. 2019). It is also possible that the injection wound suffered by intrahemocoelically injected insects was responsible for altering the physiological state of the insect, resulting in the maintenance of egg hatch rate. Future experiments measuring *G. mellonella* fecundity, egg hatch rate, egg size, and egg AMP expression after per os and intrahemocoelic injection of EPEC with sham injected and untreated controls may be able to elucidate this phenomenon. Pupal mass did not predict fecundity or egg hatch rate, which is consistent with the results from insects injected intrahemocoelically at the sublethal doses of EPEC, indicating that reproduction was prioritized over growth.

**EPEC Virulence Compared to Other Bacteria**

The ∆esN mutant of EPEC was approximately 3 times less virulent than the wild type (Table 1), indicating that the T3SS contributes to the virulence of EPEC in *G. mellonella*. This was consistent with the results of Leuko and Raivio (2012), who also found the ∆esN mutant to be less virulent than the wild type EPEC in *G. mellonella* but did not discuss it in detail. Some of the effectors secreted by the EPEC T3SS inhibit host immune responses. Macrophage phagocytosis is inhibited by EPEC effectors: EspB (*E. coli* secreted protein B), EspF, EspJ, and EspH (Dong et al. 2010, Iizumi et al. 2007, Marchès et al. 2008, Quitard et al. 2006). Phagocytosis is an evolutionarily conserved immune response in insects and vertebrates (Melcarne et al. 2019). Phagocytosis of EPEC by *G. mellonella* hemocytes may similarly be inhibited by these effectors. The nuclear factor κB (NF-κB) transcription factors are inhibited by EPEC effectors: Tir (translocated intimin receptor), NleB (non-locus of enterocyte effacement encoded protein B), NleC, NleD, NleE, NleH in HeLa cells and mice (Baruch et al. 2011; Gao et al. 2009, 2013; Nadler et al. 2010; Pearson et al. 2011; Ruchaud-Sparagano et al. 2011). In insects, the activation of NF-κB is responsible for inducing the expression of AMPs after upstream activation of the Toll and Imd pathways (Valanne et al. 2011). NF-κB in *G. mellonella* may be inhibited by these effectors and result in reduced AMP expression and increased EPEC survival. The inability of the ∆esN mutant to secrete these effectors may be responsible for the reduced virulence observed and can be tested in a future experiment by quantifying circulating bacteria over time and AMP expression post-injection. However, EPEC attachment and injection of effectors into insect cells have not yet been definitively demonstrated to date. Future experiments are required to determine which insect cell types are targeted by EPEC and the specific roles of EPEC effectors in *G. mellonella*.

The DH5α strain of *E. coli* lacks the locus of enterocyte effacement (LEE) that encodes the T3SS and some of its secreted effectors found in EPEC that are necessary for pathogenesis on the intestinal epithelial cells of vertebrates (Chart et al. 2000, Croxen et al. 2013). DH5α was 2.59 × 10³ times less virulent in *G. mellonella* than the wild type EPEC and 8.69 × 10² times less virulent than the ∆esN mutant of EPEC (Table 1), which indicates that there are factors independent of the T3SS that were responsible for most of the virulence in *G. mellonella*. The mechanism behind the low virulence of DH5α compared to the wild type and mutant EPEC in *G. mellonella* remains unknown. DH5α does not express siderophores enterobactin and aerobactin typically found in pathogenic strains of *E. coli*, which acquire iron that are essential for bacterial replication from host iron transport and storage molecules (Chart et al. 2000, Law et al. 1992). DH5α is vulnerable to attack by vertebrate complement proteins due to the inability to express long-chained LPS typically found on the surface of pathogenic *E. coli* that reduce the accessibility of the outer membrane (Chart et al. 2000). Insect AMPs also require membrane access for lytic activities (Bulet et al. 1999). It is possible that the lack of siderophores and long-chained LPS reduced the ability of DH5α to replicate and survive in *G. mellonella*, resulting in reduced virulence compared to EPEC. Future experiments could explore the roles of siderophores and long-chained LPS in EPEC virulence in *G. mellonella*.

In *G. mellonella*, EPEC was 8.88 times less virulent than the entomopathogen *P. rettgeri* but more than 2.36 × 10² times more virulent than the soil bacterium *B. clausii* (Table 1), indicating that EPEC could be considered a moderately virulent pathogen to *G. mellonella*. However, as the per os experiment indicated above, EPEC could not efficiently invade the hemocoel from the gut, making its pathogenicity low and opportunistic.

**Characterization of *G. mellonella* Immune Responses**

Typical insect immune responses against bacteria present in the hemolymph include melanization, hemolymph coagulation, nodulation, phagocytosis, and AMP production (Cytrynska et al. 2016). Aside from AMP production, all of the above immune responses were observed in *G. mellonella* after intrahemocoelic EPEC injection whereas these immune responses were absent in the control larvae injected with Ringer’s (Fig. 8), indicating that *G. mellonella* is capable of recognizing EPEC as foreign and mount appropriate immune responses against it. Unfortunately, no discernable differences in AMP levels were detected by SDS–PAGE in a preliminary experiment, possibly due to interference from the presence of other low molecular weight proteins. The expression of AMPs in *G. mellonella* is known to increase after microbial challenge (including *E. coli*) and the repertoire of expressed AMPs differ depending on the type of microbe involved (Mak et al. 2010). Future experiments using RT-qPCR could examine individual AMP expression specifically and may be able to detect EPEC-induced changes in AMP expression in *G. mellonella*. The melanized particles observed are likely formed by the deposition of melanin around EPEC, though this could not be confirmed since no green fluorescence could be detected through the melanin. Sectioning of the melanized particles in future experiments is required to determine the presence of EPEC.

**Quantification of Circulating Hemocytes, Nodules, Melanized Particles, and EPEC**

The reduction in the number of circulating hemocytes (hemocytopenia) was only observed in *G. mellonella* larvae after the
injection of viable EPEC, while Ringer’s and HK-EPEC did not induce hemocytopenia relative to the sham-injected control (Fig. 9), indicating that EPEC replication in the hemocoele is required to induce hemocytopenia. Hemocytopenia is a reaction to microbial infection in G. mellonella and is likely mediated by ENF family cytokines such as plasmatocyte-spreading peptide (PSP) and growth-blocking peptide (GBP) (Gagen and Ratcliffe 1976). The cytokines were discussed previously in the context of larval growth inhibition following EPEC challenge. Another role of PSP and GBP in insect immunity is the stimulation of plasmatocyte spreading, which increases plasmatocyte adhesion and enhances phagocytosis, nodulation, and encapsulation (Strand et al. 2000). An unidentified plasmatocyte depletion factor, likely an ENF cytokine, was found to be released from G. mellonella hemocytes following bacterial challenge in a dose-dependent manner resulting in plasmatocyte-spreading and reduced number of circulating plasmatocytes (Chain and Anderson 1983). Plasmatocytes make up approximately 60–70% of the total number of circulating hemocytes in G. mellonella (G. Wu et al. 2016a). Plasmatocyte-spreading mediated by ENF cytokines in response to EPEC replication in the hemolymph was likely responsible for most of the hemocytopenia observed. The sharp decline in hemocyte count between 3–6 h post-injection with EPEC coincided with the sharp decline in the number of circulating EPEC, suggesting the removal of EPEC from the hemolymph by nodulation (Figs. 9 and 10). Both plasmatocytes and granulocytes participate in nodulation and most nodules in G. mellonella leave circulation and attach to the surfaces of tissues in the hemocoele (Ratcliffe and Gagen 1976). Plasmatocytes not participating in nodulation could be attached to tissue surfaces in the hemocoe and out of circulation (sessile). Hemolymph coagulation likely contributes to hemocytopenia by immobilizing hemocytes along with EPEC. Recovery from hemocytopenia between 6–48 h post-injection with EPEC coincided with the clearance of EPEC from the hemolymph, indicating the restoration of homeostasis post-infection, likely by sessile plasmatocytes re-entering circulation and the production of new hemocytes (hematopoiesis). The similarity in the microaggregation of circulating hemocytes between treatments and between time points suggests that the hemocytes found in circulation were not different in their adhesion. In larvae injected with EPEC, hemocytes remaining in circulation likely represent un-activated plasmatocytes, granulocytes, and non-adhesive hemocytes (i.e., oenocytoids, sperulocytes, and prohemocytes) in the hemolymph. Nodulation was an immune response against EPEC, indicated by the appearance of nodules in the hemolymph (3–6 h post-injection) that coincided with the sharp decline in the number of circulating EPEC (Fig. 10). The majority of nodules were likely out of circulation and attached to the surfaces of tissues (Ratcliffe and Gagen 1976). Nodule attachment to tissue surfaces likely explains the absence of nodules in the hemolymph at 48 h post-injection (Fig. 10). Absence of hemocytopenia and the absence of nodules in the hemolymph in larvae injected with Ringer’s, HK-EPEC, and sham-injected larvae indicate that nodulation was not significantly induced by these treatments. Phagocytosis by hemocytes is likely sufficient in clearing HK-EPEC from the hemolymph without the involvement of nodulation since dead EPEC cannot replicate. Melanization was an immune response in the hemolymph against EPEC and HK-EPEC but not against Ringer’s. This is expected since both viable and dead EPEC present PAMPs and can be recognized by insect PRRs and induce melanization (e.g., LPS, which is heat-stable). Viable EPEC induced more melanization per larva and in more larvae than HK-EPEC due to EPEC replication. However, it is also possible that the melanized particles found in the single insect injected with HK-EPEC were, by chance, remnants of a prior infection, since melanin remains in the hemocoel permanently post-formation (Hillyer 2016). In larvae injected with EPEC, the appearance of melanized particles (3–6 h post-injection) coincided with the sharp decline in the number of circulating EPEC, suggesting that the melanized particles are likely the results of melanin deposition around EPEC (Fig. 10). Melanization associated with the injection wound indicates that all insects are indeed capable of melanization. This was expected since melanization contributes to the hardening of the clot (Rowley and Ratcliffe 1978).

At the early stage of EPEC infection (1–3 h post-injection), the number of circulating EPEC in the hemolymph sharply increased by a factor of approximately 8 (Fig. 10). The doubling time of EPEC in LB medium at 30°C, the same incubation temperature of G. mellonella, was determined to be 38 min. Exponential increase by a factor of 8 took 114 min in LB medium but 180 min in G. mellonella, indicating that G. mellonella hemolymph is not optimal for EPEC growth. The number of circulating EPEC may be reduced by early immune responses such as phagocytosis, nodulation, melanization, and hemolymph coagulation, all of which can be activated within minutes of microbial exposure (Gagen and Ratcliffe 1976, Ratcliffe and Gagen 1976). Despite these early immune responses, the insects were unable to control EPEC replication in the hemolymph between 1–3 h post-injection, evident by the increasing number of circulating EPEC. However, by 3–6 h post-injection the decline in the number of circulating EPEC indicates that the rate of EPEC clearance exceeded the rate of EPEC replication. The immune responses at this point likely involved the expression of AMPs and other immune-related proteins in addition to the early cellular and humoral immune responses mentioned above. Cecropin-A, peptidoglycan recognition-like protein-B, and prophenoloxidase-activating proteinase-1 (PAP-1) were reported to have increased expression in G. mellonella at 6 h post-injection with C. albicans (Sheehan and Kavanagh 2018). Cecropin-A is an AMP that is effective against bacteria, including multidrug resistant Acinetobacter baumannii and P. aeruginosa (Lee et al. 2015). Peptidoglycan recognition proteins (PGRPs) function as PRRs and induce AMP expression and melanization after pathogen detection (Iketani and Morishima 1993, Yoshida and Ashida 1986). PAP-1 converts the inactive prophenoloxidase (PPO) to active phenoloxidase (PO) during melanization (Zou et al. 2005). The protein expression profile of G. mellonella post-infection with EPEC is unknown and requires future experiments to investigate and is likely different from the expression profile post-infection with C. albicans, while similarly involve the expression of AMPs and other immune-related proteins enhancing EPEC clearance. The number of circulating EPEC did not change between 6–24 h post-injection, indicating that the rate of EPEC replication matched the rate of EPEC clearance. Compared to 3–6 h post-injection, this could be due to either increased EPEC replication rate, decreased EPEC clearance rate, or a combination of both. The mechanism behind this is unknown and requires further experimentation to determine. Some EPEC may have re-entered the hemolymph after escaping from nodules and coagulum, increasing the apparent replication rate. It is possible that nodulation becomes less efficient in clearing bacteria at lower densities where the bacteria were scattered and do not form aggregates. Complete clearance of EPEC from the hemolymph occurred between 24–48 h post-injection, likely due to the expression of additional AMPs and immune-related proteins at this later stage of infection. The expression of PRRs (PGRP-LB and hemolin) and AMPs (gloverin, cecropin D-like peptide, and moricin-like peptide B) were increased in G. mellonella larvae.
The intrahemocoelic injection of Ringer’s, HK-EPEC, and the injection injury did not kill any G. mellonella or delay pupation. Interestingly, EPEC was cleared from the hemolymph by 48 h post-injection at which point all insects were alive at the time of hemolymph collection, while 60% mortality (12 out of 20) was observed among the undisturbed insects by 20 d post-injection. Furthermore, out of the 12 dead insects, only 1 died from visible bacteremia whereas the remaining 11 died with no viable EPEC in the hemolymph. This indicates that there are at least 3 potential outcomes of EPEC infection in G. mellonella larvae at the approximate LD₅₀: (1) The larva clears all EPEC from the hemolymph and completes development into an adult. (2) The larva clears all EPEC from the hemolymph but dies anyway, likely due to irrecoverable damage to the larva by EPEC and the immune responses. (3) The larval immune responses fail to control EPEC replication and the larva succumbs to death by septicemia. Future experiments are required to determine the intricate dose-dependent effect of EPEC on the infection outcome. Outcome (2) likely represents moribund larvae that were able to remain alive for up to 20 d post-injection before finally succumbing to death. As EPEC dose increases beyond the LD₅₀, outcome (3) is expected to become increasingly prevalent and vice versa for outcome (1).

Conclusions
EPEC causes disease in G. mellonella when injected intrahemocoelically. Disease severity is dose-dependent and manifests as increased mortality, decreased survival time, delayed pupation, decreased pupal mass, and increased pupal duration. Disease symptoms can be used as metrics for the measure of EPEC virulence in G. mellonella and are likely due to a combination of septicemia and immunopathology. EPEC was not pathogenic per os, likely due to inefficient translocation from the insect gut to the hemocoel where it is virulent. The T3SS was partially responsible for EPEC virulence in G. mellonella, possibly through the inhibition of phagocytosis and AMP expression by secreted effectors, allowing increased EPEC survival in the hemocoel. There were unknown factors independent of the T3SS in EPEC responsible for most of the virulence, which may include siderophore and long-chained LPS expression.

After intrahemocoelic injection of EPEC, G. mellonella larvae showed typical insect anti-bacterial immune responses including melanization, hemolymph coagulation, nodulation, and phagocytosis. Hemocytopenia was temporarily induced in G. mellonella by EPEC, likely through plasmatocyte-spreading and nodulation. The subsequent recovery from hemocytopenia was likely through the release of sessile hemocytes and hemolysis. The immune responses of G. mellonella were unable to control EPEC replication at the early stage of infection but were eventually able to clear EPEC from the hemolymph, likely through a combination of phagocytosis, nodulation, melanization, and increased AMP and immune-related protein expression. The clearance of EPEC did not guarantee insect survival, likely due to irrecoverable damage from EPEC and the immune responses.

Overall, this study provided insights into EPEC virulence and pathogenesis in G. mellonella and identified areas of future research using this system.

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Author Contributions
RYY: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing. BAK: Conceptualization, funding acquisition, methodology, resources, supervision, writing – review and editing.

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