Characterization and mode of action analysis of black soldier fly \((Hermetia illucens)\) larva-derived hemocytes

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Abstract With the growing importance of the black soldier fly \((Hermetia illucens)\) for both sustainable food production and waste management as well as for science, a great demand of understanding its immune system arises. Here, we present the first description of the circulating larval hemocytes with special emphasis on uptake of microorganisms and distinguishing hemocyte types. With histological, zymographic, and cytometric methods and with a set of hemocyte binding lectins and antibodies, the hemocytes of \(H. illucens\) are identified as plasmatocytes, crystal cells, and putative prohemocytes. Total hemocyte counts (THC) are determined, and methods for THC determination are compared. Approximately 1100 hemocytes per microliter hemolymph are present in naive animals, while hemocyte density decreases dramatically shortly after wounding, indicating a role of hemocytes in response to wounding (and immune response in general). The determination of the relative abundance of each hemocyte type (differential hemocyte count, DHC) revealed that plasmatocytes are highly abundant, whereas prohemocytes and crystal cells make up only a small percentage of the circulating cells. Plasmatocytes are not only the most abundant but also the professional phagocytes in \(H. illucens\). They rapidly engulf and take up bacteria both in vivo and in vitro, indicating a very potent cellular defense against invading pathogens. Larger bioparticles such as yeasts are also removed from circulation by phagocytosis, but slower than bacteria. This is the first analysis of the potent cellular immune response in the black soldier fly, and a first toolbox that helps to identify hemocyte (types) is presented.

Key words black soldier fly \((Hermetia illucens)\); cellular immunity; crystal cell; hemocyte types; immunity of economically important insects; microbe phagocytosis; plasmatocyte

Introduction

Within the emerging field of sustainable circulatory economies, the black soldier fly \textit{Hermetia illucens} (L. 1758) plays a key role in the conversion of organic waste into biomass. This is reflected by the recent enormous output of publications on economic values, mass breeding, waste management and use as livestock feed (reviewed e.g., in Baragan-Fonseca et al., 2017; Müller...
lated genes in may explain the remarkable expansion of immune re-
in contact with potential pathogens. A potent immune elomi, 2017), and therefore face a high risk to come (e.g., organic waste, excrements, cadavers) (Wang & Shelomi, 2017). Black soldier fly larvae (e.g., PGRPs, AMPs, GNBPs) (Vogel et al., 2018; Zhan et al., 2020). By the characterization of antimicrobial peptides (AMPs) and other components and functions of the immune system the basis was established for a comprehensive understanding of the immune system of this emergent insect model (Park et al., 2014; Park et al., 2015; Zdybicka-Barabas et al., 2016; Elhag et al., 2017; Park & Yoe, 2017; Jung et al., 2018; Vogel et al., 2018; Shin & Park, 2019). The second branch of the innate immune system is the cellular immunity which comprises mainly hemocytes, and acts together with the fast humoral response (e.g., phenoloxidase-mediated melanization and constitutively expressed AMPs) as a first line of defense toward invading threats (Lavine & Strand, 2002; Haine et al., 2008).

Hemocytes are freely circulating and/or sessile cells in the hemocoel, which play a crucial role in normal development and immune response (reviewed in Lavine & Strand, 2002; Ribeiro & Brehélin, 2006; Strand, 2008; Hillyer, 2016). Morphological and functional criteria allow to distinguish certain hemocyte types. With a growing number of descriptions and interpretations the number of proposed insect hemocyte types ever increased, until Jones (1962) and Price and Ratcliffe (1974) simplified the nomenclature by integrating subforms into major hemocyte types. Within these main classifications, subtypes may occur in form of peculiar morphology (e.g., Yeager, 1945; Jones, 1962) or gene expression patterns (Cattenoz et al., 2020).

The main hemocyte types in insects are therefore pro-
hemocytes, plasmatocytes, granular cells, spherule cells, and oenocytoids or crystal cells (see for comparison and nomenclature of insect hemocyte types Jones, 1962; Price & Ratcliffe, 1974; Gupta, 1979; Ribeiro & Brehélin, 2006; Strand, 2008; Hillyer, 2016). It is commonly accepted that the hemocyte types, which perform phagocytosis in response to invading pathogens or in order to remove cell debris are named either granular cells or plasmatocytes (reviewed in Ribeiro & Brehélin, 2006). Some authors state that plasmatocytes of distinct species are not phagocytically active. Other hemocyte types are specialized for example on synthesis and release of (pro-)phenoloxidase (Pro-)PO, the key component of the melanization cascade. (Pro-)PO releasing hemocytes are called either oenocytoids (in most lepidopteran species and other insects) or crystal cells (in many dipteran and few lepidopteran species; with eponymous crystalline inclusions) and can easily be distinguished by both their appearance and—in the case of crystal cells—intracellular components. Another commonly identified type is the prohemocyte, usually described as progenitor cell, which is able to divide and differentiate into other hemocyte types. These are usually small cells with a high nucleus-to-cytoplasm ratio (Beaulaton & Monpeyssin, 1976; Gupta, 1979; Gardiner & Strand, 2000). Since tracing hemocyte lineages remains difficult due to the lack of powerful genetic tools (which is valid for virtually all species but Drosophila melanogaster), insect hemocyte classification still relies mostly on morphology and function.

Most insect species, however, do not necessarily possess every hemocyte type described above. A comparison of the cellular components of the hemolymph of several cyclorrhaphan Diptera species revealed differential blood pictures for each species. Chrysomya megacephala for example contains prohemocytes, plasmatocytes, granulocytes, oenocytoids, and sperulocytes, while Musca domestica contains additionally adipohemocytes, and Sarcophaga ruficornis contains additionally vermicytes but no adipohemocytes (Pal & Kumar, 2014). Even on a narrow taxonomic level, the blood cell composition may differ. D. melanogaster contains prohemocytes, plasmatocytes, crystal cells, and (inducible) lamellocytes (Rizki & Rizki, 1980), while another species from the same genus—D. ananassae—lacks lamellocytes but instead forms multinucleated hemocytes in response to parasitosis (Márkus et al., 2015). These examples underline that close phylogenetic relationship between species is no prerequisite for shared hemocyte compositions.

The cellular components in the hemolymph of H. illucens were briefly described by Zdybicka-Barabas et al. (2016) based on light microscopic observations of live cells. Since no specific markers for H. illucens hemocytes are known to date, we examined binding properties of general cell component markers, lectins, and antibodies generated against targets of other organisms, stained hemocytes with histological methods, and examined their ability to phagocytize potentially harmful particles. Based on these data, we classified the hemocytes into three major types and termed them according to the D. melanogaster hemocyte nomenclature. One of these types rapidly phagocytized both living and dead microorganisms. With the high number of phagocytic active hemocytes present, we conclude that the cellular immune reaction of H. illucens is a potent line of defense against
invasive pathogens, which together with the expanded set of antimicrobial peptide and pattern recognition receptors coding genes forms an efficient immune system.

**Materials and methods**

**Hermetia illucens rearing**

*Hermetia illucens* larvae were obtained from the pilot breeding plant from Bio.S GmbH, Grimma, Germany, and reared in the Institute of Zoology, TU Dresden. Larvae were kept crowded plastic containers with vented lids at 23°C, and fed ad libitum with moistened chicken feed (Deuka All Mash-L, Deutsche Tierernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). The larval molt several times before reaching the prepupa stage (wandering instar), which are darker than younger stages and cease food uptake. Each experiment was conducted with larvae of the last stage before molting to the wandering stage, with an approximate body weight between 100 and 250 mg. We designated this stage as last feeding instar (LFI). We avoid enumeration of larval stages due to discrepancies between the numbers of molts, and consequently the numbers of larval stages, described by different authors (cf. six molts: Schremmer, 1984; Kim *et al.*, 2010; Barros *et al.*, 2019; Gligorescu *et al.*, 2019; five molts: May, 1961; four molts: Hall & Gerhard, 2002). Prior to each experiment, the animals were washed extensively with tap water, followed by rinsing with demineralized water and 70% (v/v) ethanol and air dried.

**Total hemocyte count**

For the determination of the total hemocyte count (THC), *H. illucens* larvae were heat-fixed by immersion in a 60°C water bath for 1 min prior to hemocyte harvesting. The heat-treatment immediately immobilized the larvae completely. Since the larvae did not recover from the heat-shock and blackened within 48 h heating to 60°C for 1 min was fatal (*n* = 10 larvae observed over 96 h, data not shown). The heat-treatment inactivated the ability of hemocytes to form pseudopodia whereas staining with trypan blue (adding one part of 0.4% (w/v) trypan blue in PBS to one part of hemolymph, 5 min incubation) revealed that all hemocytes except crystal cells did not stain blue, indicating that the cells were still alive (own observations, data not shown). Due to the effect on spreading and pseudopodia formation, heat-treatment was only used for THC determination. Hemolymph was collected by cutting the dorsal epidermis of the sixth abdominal segment, and 10 μL of the oozing hemolymph was immediately applied on a Neubauer improved counting chamber. Hemocytes in a total volume of 0.04 μL were counted with phase contrast and 400× magnification with a Leica DM LS microscope (Leica Microsystems GmbH, Wetzlar, Germany). Both naive larvae and larvae wounded by pricking with a sterile needle at the posterior abdomen (1, 2, or 24 h prior to THC counts) were examined for total hemocyte concentration in order to determine changes in circulating hemocyte numbers after wounding. Additionally, the THC of non-heat-fixed animals was determined to compare both methods.

**Collection of live hemocytes and monolayer preparation**

Hemocytes were obtained by cutting the dorsal side of the sixth abdominal segment of *H. illucens* LFI-larvae with a fine scissor. The oozing hemolymph was immediately aspirated with a microliter pipette and either diluted in appropriate medium or used undiluted.

To obtain hemocyte monolayers the hemolymph was diluted 1 : 2 in Schneider’s insect cell culture medium (Thermo Fisher, Waltham, USA) with few crystals of phenylthiourea (PTU) (Schneider’s+PTU) and placed on multi well glass slides. Hemocytes were allowed to settle for 20 min at room temperature in a moist chamber. The medium was then discarded, the hemocytes rinsed briefly with phosphate buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1.8 mmol/L KH₂PO₄, pH 7.4), fixed for 5 min with 4% paraformaldehyde in PBS, pH 7.4, and rinsed three times with PBS. For most experiments, the monolayers were used immediately after the preparation. The cells, however, can also be stored at −20°C for subsequent antibody- or lectin-labeling.

**Giemsa staining of hemocytes**

Hemocyte monolayers were prepared as described above (Collection of live hemocytes and monolayer preparation), rinsed with water and air-dried. After fixation with pure methanol for 1 min at room temperature the cells were stained with freshly prepared Giemsa solution (Giemsa stock solution, Merck KgaA, Darmstadt, Germany, diluted 1 : 5 in water) for 10 min. After rinsing with 0.1 mol/L potassium phosphate buffer (pH 8.0) the cells were shortly rinsed with water, air dried, dehydrated in xylol (100%) over night, and mounted with DPX (Merck KgaA, Darmstadt, Germany).

**Supravital staining of hemocytes**

Lysosomes in live hemocytes were visualized with neutral red staining (method modified acc. to Richard-
son et al., 2018). Freshly collected hemolymph was diluted 1:10 in neutral red staining solution (125 μmol/L neutral red (Merck KgaA, Darmstadt, Germany) in Schneider’s+PTU) and placed on glass slides. Hemocytes were allowed to settle in a moist chamber for 20 min at room temperature, and staining visualized by bright field and phase contrast microscopy at 630× magnification with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany).

**In situ zymography for determination of peroxidase and (pro-)phenoloxidase activity**

Hemolymph was collected as described above (Collection of live hemocytes and monolayer preparation) and diluted 1:2 in Schneider’s insect cell culture medium. The cell suspension was placed on a multiwell glass slide and allowed to settle for 5 or 10 min in a moist chamber under microscopic control. Then, the fluid was discarded, the cells rinsed two times with 50 mmol/L Tris (pH 7.5) and immediately fixed with precooled (−20°C) acetone at −20°C for 4 min. After three rinses with 50 mmol/L Tris (pH 7.5) at room temperature the chromogenic substrate solution (2.33 mmol/L 3,3′-diaminobenzidine (DAB), 128 μmol/L hydrogen peroxide, 150 mmol/L sodium azide dissolved in 50 mmol/L Tris, pH 7.5) was added for 30 min. In order to differentiate between peroxidase and phenoloxidase activity, controls were performed by either adding PTU crystals to the chromogenic substrate solution (inhibition of phenoloxidases by PTU, cf. Ryazanova et al., 2012; inhibition of peroxidases by sodium azide) or by incubation in chromogenic substrate solution with PTU and without sodium azide (inhibition of phenoloxidases, no inhibition of peroxidases).

**Fluorescence labeling of hemocytes**

Hemocyte monolayer were prepared and paraformaldehyde fixed as described above (Collection of live hemocytes and monolayer preparation). All fluorescence-labeled hemocytes were mounted in Fluoromount G (Thermo Fisher Scientific, Waltham, USA).

**Immunofluorescence labeling** Parafomaldehyde fixed hemocytes were rinsed with PBS, and blocked with 5% (v/v) normal goat serum (NGS), 3% (w/v) bovine serum albumin (BSA) in PBS (pH 7.4). Thereafter, the cells were labeled with rabbit anti- D. melanogaster Papulin immune serum (Campbell et al., 1987, from the laboratory stock of H. Gutzeit), diluted 1:250 in 5% (v/v) NGS, 3% (w/v) BSA in PBS (pH 7.4) at 4°C over night. After three rinses with PBS, DyLight 488 conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, USA; RRID: AB_2336402; diluted 1:2000 in 5% (v/v) NGS, 3% (w/v) BSA in PBS (pH 7.4) was added for 1 h at room temperature. Then, the cells were rinsed twice with PBS. Nuclei were stained with 360 nmol/L 4′, 6-diamidino-2-phenylindole (DAPI) in 0.1% Tween 20® in PBS (PTW) for 30 min at room temperature, followed by two rinses with PBS. For additional labeling with peanut agglutinin (PNA), TRITC conjugated PNA (Sigma Aldrich, St. Louis, USA) was diluted 1:500 in the secondary antibody solution.

Labeling of mitotic hemocytes with rabbit anti-phosphorylated histone H3 (Ser10) polyclonal antibody (anti-PHH3; Merck KgaA, Darmstadt, Germany; RRID: AB_310177; diluted 1:200) was performed as described above with following modification: the hemocytes were permeabilized with 0.1% (v/v) Triton X-100 in 1% (w/v) BSA in PBS for 5 min and rinsed four times with PBS prior to blocking. Relative number of anti-PHH3 positive hemocytes from n = 7 animals with at least 500 hemocytes per animal was determined by fluorescence microscopy (total hemocytes analyzed n = 8752).

Additionally, several antibodies known from previous work to bind to hemocytes of other insect species were tested for their ability to bind to hemocytes: rabbit antihorseradish peroxidase (Sigma Aldrich, St. Louis, USA; RRID: AB_261181; diluted 1:500), rabbit anti-Bombyx mori phenoloxidase (Iwama & Ashida, 1986, diluted 1:500), mouse monoclonal anti-Manduca sexta Neuroglian (Nardi, 1993, RRID AB_10804674, undiluted hybridoma supernatant) (Table S2).

**Lectin labeling** The lectins screened for binding to hemocytes were: FITC-conjugated Datura stramonium agglutinin (DSA, Vector Laboratories, Burlingame, USA), TRITC-conjugated Helix pomatia agglutinin (HPA, Sigma Aldrich, St. Louis, USA), FITC-conjugated Artocarpus integrifolia lectin (Jacalin, Vector Laboratories, Burlingame, USA), FITC-conjugated Lens culinaris agglutinin (LCA, Vector laboratories, Burlingame, USA), FITC-conjugated Lycopersicon esculentum agglutinin (LEA, Vector Laboratories, Burlingame, USA), TRITC-conjugated Maclura pomifera agglutinin (MPA, Sigma Aldrich, St. Louis, USA), FITC- or TRITC-conjugated Arachis hypogaea agglutinin (peanut agglutinin, PNA, Sigma Aldrich, St. Louis, USA), FITC-conjugated Pisum sativum agglutinin (PSA, Vector laboratories, Burlingame, USA), FITC-conjugated Glycine max agglutinin (soy bean agglutinin, SBA, Vector laboratories, Burlingame, USA), FITC-conjugated Solanum tuberosum agglutinin (STA, Vector Laboratories, Burlingame, USA), FITC-conjugated Ulex europaeus agglutinin I (UEA-I, Vector Laboratories, Burlingame, USA), and FITC- or TRITC-conjugated Triticum vulgaris agglutinin.
Lectin staining was carried out on PFA fixed hemocytes. The first screen to test for lectin binding in general was performed without the use of detergent. For some lectins tested positive in the first screen, the hemocytes were either permeabilized with 0.1% (v/v) Triton X-100, 1% (w/v) BSA in PBS for 5 min prior to blocking or not in order to differentiate between cell surface labeling and labeling of intracellular structures. This detergent treatment did not interfere with or change the pattern of PNA, MPA, or WGA staining, whereas the HPA signal was very weak when Triton X-100 was used. The monolayers were then blocked with 5% (v/v) normal goat serum (NGS), 3% (w/v) bovine serum albumin (BSA) in PBS (pH 7.4) as described for antibody labeling. Each lectin was diluted 1:200 in 3% BSA-PBS prior to staining the cells at 4°C overnight. The hemocyte monolayers were then rinsed twice with PBS, incubated with 360 nmol/L DAPI in PTW for 30 min at room temperature, and rinsed twice with PBS.

**F-actin labeling** For labeling of filamentous actin (F-actin), the paraformaldehyde fixed hemocytes were permeabilized with 0.1% (v/v) Triton X-100®, 1% (w/v) BSA in PBS for 5 min, rinsed twice with PBS, preincubated with 1% BSA (w/v) in PBS for 30 min, and incubated with Rhodamine conjugated Phalloidin (Thermo Fisher Scientific, Waltham, USA; RRID: AB_2572408), diluted 1:200 in 1% (w/v) BSA in PBS for 1 h.

**Live imaging of hemocytes and in vitro bacteria uptake by hemocytes**

For the examination of freshly harvested hemocytes 200 μL Schneider’s + PTU was placed in the center of a 35 mm glass bottom dish (MatTek Life Sciences, Ashland, USA). Hemolymph of a single larva (approximately 20 μL) was harvested as described above (Collection of hemocytes and monolayer preparation) immediately added and the hemocytes were examined with a Zeiss Axiovert.A1 microscope (Carl Zeiss Microscopy, Jena, Germany).

For live imaging of immobilization and uptake of live bacteria by hemocytes, 200 μL Schneider’s insect medium containing 2 mmol/L PTU, 125 μg/mL Amphotericin B, 100 μg/mL Erythromycin, 20 μg/mL Ciprofloxacin was cooled on ice. Fifty microliters of hemolymph (pooled from five individuals) were collected in the medium on ice and thoroughly mixed by pipetting. The whole volume (250 μL) was transferred in a μ-slide 8 well chamber (ibidi GmbH, Gräfelfing, Germany). Live GFP-expressing *Bacillus subtilis* (TMB4131, W168 lacA::P_{reg}^sfGFP, PMID: 29118374, Popp et al., 2017) from a fresh over night culture were added to final concentrations of $2 \times 10^6$, $6 \times 10^6$, or $2 \times 10^7$ bacteria per mL, respectively. The cells were imaged for three hours with 8–30 s steps between each photograph with a Zeiss ObserverZ.1 microscope (Carl Zeiss Microscopy, Jena, Germany).

**Fluorescence labeling of microorganisms**

**FITC labeling of microorganisms** Overnight cultures of bacteria (*E. coli* BL21 [DE3], *Bacillus subtilis*, both from the microorganism collection of the Institute for Microbiology, TU Dresden) were grown over night in LB-Miller medium at 30°C. *Saccharomyces boulardii* was isolated from a commercial medical product (Perenterol® Forte, MEDICE Arzneimittel Pütter GmbH & Co. KG., Iserlohn, Germany), overnight cultures were grown in yeast extract peptone dextrose medium (YPED) at 30°C. The microorganisms were washed twice with 0.7% (w/v) NaCl and killed by autoclaving at 121°C. Thereafter, the microorganisms were rinsed twice with sterile 0.1 mol/L sodium bicarbonate buffer, pH 9.0, adjusted to $1 \times 10^{10}$ cells/mL (bacteria) or $1 \times 10^9$ cells/mL (*S. boulardii*), and labeled with 0.0015% (w/v) fluorescein isothiocyanate (FITC, Sigma Aldrich, St. Louis, USA) in sterile 0.1 mol/L sodium bicarbonate buffer, pH 9.0 for 30 min at room temperature. Thereafter, excessive FITC was removed by three rinses with sterile 0.7% (w/v) NaCl. Labeled microorganisms were stored in 0.7% (w/v) NaCl at 4°C in the dark.

**pHrodo labeling of microorganisms** *E. coli* K12 D31 (Monner et al., 1971) were grown over night in LB-Miller medium, washed twice with 0.7% (w/v) NaCl, followed by washing with demineralized water. The bacteria were then lyophilized, and labeled with 0.5 mmol/L pHrodo™ iFL Green STP Ester (Invitrogen™ Thermo Fisher, Waltham, USA) according to manufacturer’s instructions. Similarly, *S. boulardii* were grown over night in YEPD, washed twice with 0.7% (w/v) NaCl, and demineralized water. The microorganisms were then lyophilized, and conjugated with 0.5 mmol/L pHrodo™ iFL Red STP Ester (Invitrogen™ Thermo Fisher, Waltham, USA) according to manufacturer’s instructions.

pHrodo-labeled microorganisms were lyophilized and stored in 0.25 mg aliquots at $-20°C$ until used.

pH-dependent fluorescence of the conjugated microorganisms was determined before each experiment by adding PBS adjusted to specific pH (pH 7.5, pH 6.0, pH...
4.5) to the labeled microorganisms and examination of the fluorescence activation under a fluorescence microscope. For pHrodo Green-labeled *E. coli*, no fluorescence was detectable in neutral to slightly basic pH (pH 7.5) and slightly acidic pH (pH 6.0). At pH 4.5, however, the labeled microorganisms exhibited a strong fluorescence. pHrodo Red-labeled yeasts exhibited detectable fluorescence at each pH tested under a fluorescence microscope due to unknown reasons. In live imaging of phagocytic hemocytes by confocal laser scanning microscopy, however, we observed mostly nonfluorescent yeasts outside the hemocytes and fluorescent yeast particles incorporated by the hemocytes.

**Determination of phagocytosis in vivo**

*In vivo* phagocytosis ability of hemocytes was determined by injection of FITC-labeled microorganisms. In each *H. illucens* larva a total of 2 × 10⁷ (bacteria) or 2×10⁶ (*S. boulardii*) cells in 20 µL 0.7% (w/v) NaCl was injected slightly laterally to the dorsal vessel in abdominal segment 6. At defined time points hemolymph samples were collected and hemocyte monolayers prepared, fixed, and stained with Phalloidin and DAPI as described above (F-actin labeling). F-actin labeling allowed to distinguish between external and internalized microorganisms (cf. a similar method used by King & Hillyer, 2012, 2013). Internalization of microorganisms is most likely the result of phagocytosis; therefore, we refer to hemocytes with internalized microorganisms as phagocytotically active. Phagocytosis was examined by fluorescence microscopy. Hemocytes were subdivided according to their appearance and classified into nonphagocytic (no fluorescent microorganisms present within the cell) and phagocytic hemocytes (microorganisms co-localized with the cell, within the F-actin lined cytoplasm). Aggregates of more than three hemocytes were also noted but were not used for the determination of phagocytic hemocytes. At least 20 positions per slide and animal were counted under a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) with 400× magnification.

As a control of the method of phagocytosis determination, we compared the pattern of internalized bacteria in the fixed samples with that in live hemocytes after trypan blue quenching. Three animals were injected with FITC-labeled *B. subtilis* as described above. After 30 min, 10 µL of hemolymph was diluted in the same volume of Schneider’s+PTU, and 20 µL 0.4% (w/v) trypan blue in PBS (quenching of nonphagocytized *B. subtilis*) or 20 µL PBS (no quenching of fluorescence) was added for 20 min. Thereafter, the fluid was discarded, the hemocytes rinsed two times with PBS, and immediately imaged with a fluorescence microscope with FITC filter, 1000 ms exposure time.

Additionally, in vivo phagocytosis assays were performed with *E. coli* K12 D31 labeled with pHrodo Green or *S. boulardii* labeled with pHrodo Red, respectively. The fluorochromes are pH-sensitive; therefore, fluorescence signals indicate microorganism uptake by phagocytosis as the fluorochromes are activated by the acidic environment of the phagolysosomes. Animals were injected with 5 µg *E. coli* K12 D31 pHrodo Green or 5 µg *S. boulardii* pHrodo Red diluted in 50% Schneider’s+PTU, 50% 0.7% (w/v) NaCl. Hemolymph was collected after 30 min (*E. coli* K12 D31) or 180 min (*S. boulardii*) and diluted approximately 1 : 2 in Schneider’s+PTU. The cells were allowed to settle for 10 min, and immediately imaged with a Zeiss AxioObserver LSM 880 confocal laser scanning microscope.

**Differential hemocyte count**

Giemsa-stained hemocyte monolayers from *n = 6* animals were examined with a Leica DM LS microscope (Leica Microsystems GmbH, Wetzlar, Germany) with 400× magnification. A total of *n = 3303* cells were classified according to shape, size, and nucleus appearance into following types: spreading cells with lamellipodia- or filopodia-like protrusions (typically referred to as plasmocytes), small cells with sparse cytoplasm and relatively large, often elongated nucleus (prohemocytes), large round to ovoid cells without crystalline inclusions, large round to oval cells with crystalline inclusions (crystal cells), small round to oval cells without protrusions, and small round to oval cells with protrusions. A total of *n = 469* cells was additionally observed in *n = 97* aggregates, each comprising more than three cells. These cells were omitted from cell type determination.

**Imaging and graphic design**

Light microscopy was performed with a Zeiss Axioplan 2 (Carl Zeiss Microscopy, Jena, Germany) microscope with mounted ToupTek E3CMOS camera (ToupTek Photonics Co., Ltd, Hangzhou, China) or Jenoptik ProgRes SpeedXTcore5 camera (Jenoptik AG, Jena, Germany). Fluorescence and bright field microscopy was performed with a Zeiss Observer.Z1 with mounted Zeiss AxioCam 506 mono camera (both Carl Zeiss Microscopy, Jena, Germany). Confocal laser scanning microscopy was performed with a Zeiss...
AxioObserver LSM 880 and ZEN Black software V. 2.3 (both Carl Zeiss Microscopy, Jena, Germany). Fluorescence photomicrographs were adjusted for overall brightness and contrast with ImageJ V. 1.52p (Schindelin et al., 2012). Photomicrographs were adjusted for overall brightness and contrast with GIMP 2.10.14 (The GIMP Development Team, 2019, http://www.gimp.org/) and InkScape 0.92.4 (http://inkscape.org). Figure panels were assembled using GIMP 2.10.14 (The GIMP Development Team, 2019, http://www.gimp.org/) and InkScape 0.92.4 (http://inkscape.org).

Drawings were made with a digital graphic tablet (Cintiq 16, Wacom K.K., Kao, Japan) and Clip Studio Paint Pro software V 1.10.13 (Celsys Inc., Tokyo, Japan).

Cytometry

Photomicrographs of fixed and stained hemocytes were analyzed with ImageJ V. 1.52p (Schindelin et al., 2012), plugin “Measure,” for cell diameter, nucleus diameter, nucleus and cytoplasm area. For each hemocyte type, at least 10 cells from at least three animals were analyzed. The nucleus to cytoplasm ratio (N:C) was calculated as nucleus area divided by cytoplasm area (cell area minus nucleus area).

Statistics

Statistical analyses on normal distribution (Shapiro–Wilk test) and significance (Kruskal–Wallis one-way ANOVA on ranks) were performed with SigmaPlot 11 (Systat Software, San Jose, USA). Statistical analyses of correlation between body weight and THC was performed with R version 3.6.2 (R Core Team, 2014), Kendall rank correlation coefficient test.

Diagrams were created with R version 3.6.2 (R Core Team, 2014) package ggplot2 (Wickham, 2009).

Results

Total hemocyte count in larval H. illucens

Circulating hemocytes have the ability to adhere to wound sites or internal tissue. Therefore, the determination of the total hemocyte population may be influenced by several factors including the collection method. Here, the total number of hemocytes per volume hemolymph was determined in last feeding instar H. illucens. An evaluation of three methods was performed: (1) hemolymph was collected from live specimens without pretreatment (untreated), (2) larvae were heat-treated by immersion in a 60°C water bath for 1 min prior to hemolymph collection (heated), and (3) larvae were chilled on ice for 30 min prior to hemolymph collection (cooled).

Hemolymph samples of untreated larvae (n = 22 animals) contained mean ± SD 334 ± 212 hemocytes/μL, hemolymph of heat-fixed larvae (n = 56 animals) contained mean ± SD 1138 ± 588 hemocytes/μL (Fig. 1A). For cooled larvae, hemolymph volume collected was mostly low, and samples with a sufficient amount of hemolymph (10 μL) did contain very low numbers of hemocytes, which did not allow an exact determination of the hemocyte concentration (data not shown).

Additionally, we tested the possibility of a correlation between body weight and hemocyte concentration in naive heat-treated LFI-larvae. Fifty larvae with a body weight between 50 mg and 230 mg with a median body weight of 140 mg were analyzed. No significant correlation between body weight and THC occurred, indicating that the body weight does not influence the hemocyte concentration (Fig. 1B).

Next, we examined if the number of circulating hemocytes changes in response to wounding, and compared the THC of naive larvae and sterile wounded larvae 1, 2, and 24 h after wounding. Wounding significantly decreased the number of circulating hemocytes compared to naive (unwounded) larvae 1 and 2 h after wounding, but 24 h after wounding the THC was comparable to the naive status (Fig. 2; 1 h post-wounding mean ± SD 676 ± 336 hemocytes/μL, n = 35 animals analyzed; 2 h post-wounding mean ± SD 718 ± 270 hemocytes/μL, n = 34 animals analyzed, 24 h post-wounding mean ± SD 924 ± 623 hemocytes/μL, n = 51 animals analyzed).

Description of hemocyte types

Our attempt to classify the hemocyte types in H. illucens included a simplified scheme, which allowed identification of three characteristic hemocyte types by light microscopy, enzymatic activity, histological staining, and cytometry (histological and cytometric characteristics are summarized in Table 1). These were the prohemocytes, plasmocytes, and crystal cells. The nomenclature of the hemocyte types is based on the classification in D. melanogaster.

Light microscopy of live hemocytes Under the light microscope (phase contrast or DIC), most cells in freshly harvested hemolymph appeared round, and few cells appeared spindle-shaped. Cytoplasmic protrusion formation was visible in some hemocytes within a few minutes (Fig. S1). Other hemocytes contained crystalline inclusions. Few hemocytes disintegrated within a few minutes after bleeding when observed in vitro (data not shown).
### Table 1  Histological and cytometric characteristics of the larval hemocyte types.

| Hemocyte Type     | Pseudopodia | Cell shape          | Nucleus shape         | Phagocytosis | (P)PO activity | Cell diameter min–max; mean [μm] | Nucleus diameter min–max; mean [μm] | N:C min–max; mean | Giemsa cytoplasm staining |
|-------------------|-------------|---------------------|-----------------------|--------------|----------------|-------------------------------|-------------------------------------|-------------------|--------------------------|
| Prohemocyte       | −           | Round-oval          | Round to elongated    | −            | −              | 7.1–15.8; 11.9                | 4.4–10.5; 8.1                     | 0.52–2.19; 1.21   | Intense blue to purple   |
| Crystal cell      | −           | Round-oval          | Round                 | −            | +              | 13.2–27.1; 18.3                | 5.7–10.1; 7.7                     | 0.13–0.53; 0.26   | Intense purple to blueish purple |
| Plasmatocyte      | +           | Round, oval         | Round, oval or irregularly shaped | +            | −              | 10.6–26.0; 15.8                | 6.2–11.5; 8.2                     | 0.22–2.58; 0.40   | Light purple, with unstained vacuole-like structures and small pink inclusions (eosinophilic granules) |
| Plasmatocyte      | +           | Spread, with pseudopodia | Round, oval or irregularly shaped | +            | −              | 12.5–38.3; 27.5                | 7.5–13.8; 10.4                   | 0.09–0.28; 0.18   | Light purple, with unstained vacuole-like structures and small pink inclusions (eosinophilic granules) |

*Transitions between round and spread plasmatocytes may appear round with early pseudopodia formation. Symbols: −, characteristic absent; +, characteristic present.*
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Fig. 1 Total hemocyte counts of last feeding instar H. illucens. (A) Influence of heat-fixation on total hemocyte concentration. Hemolymph samples of heat-fixed larvae (heated) contained significantly more hemocytes per μL (mean ± SD 1138 ± 588 hemocytes/μL, n = 56 animals analyzed) than non-heat-fixed (untreated) larvae (mean ± SD 334 ± 212 hemocytes/μL, n = 22 animals analyzed). Asterisk depicts significant difference (Wilcoxon unpaired rank sum test, W = 1159.5, P = 1.629 × 10⁻⁹). Black dots denote outliers and grey and black dots denote each observation. Each box indicates the upper and lower quartile, whiskers indicate 1.5-fold interquartile distance, the median is depicted by a black horizontal bar. *Haemocyte count per μL indicates that aggregates of more than three cells were ommitted from the analysis. (B) Analysis of the total hemocyte counts of LFI larvae with different body weights. The hemocyte concentration does not correlate significantly with the body weight (Kendall’s z = 1.2769, tau = 0.1279, P = 0.2016). Total hemocyte counts of n = 50 heat-fixed LFI-larvae with body weights ranging from 50 mg to 230 mg were determined.

The cytoplasm of round cells with or without protrusions, as well as of spindle-shaped cells appeared granulated. Many cells, which adhered on the glass substratum flattened and a subset of these cells, formed lamellipodia and/or filopodia. These cells then exhibited a symmetrically or asymmetrically spread morphology, with an elevated central portion containing the nucleus. Granulation of the cytoplasm as seen by light microscopy changed during flattening (Fig. S1), becoming more prominent with progressing culture time, and in some cells, larger intracellular inclusions appeared (Fig. S1, cf. also Movies S1–S3). Based on these observations, granularity of the cytoplasm in H. illucens hemocytes is a weak characteristic (cf. definition of granulated insect hemocytes by Rowley and Ratcliffe in Ratcliffe & Rowley, 1981).

Giemsa-staining, cytometry, and (pro-)phenoloxidase activity of hemocytes

The hemocytes further referred to as plasmatocytes exhibited a wide variance of shapes when adhering on a glass substratum. These cells were either round or spread. They had in common that after Giemsa-staining the cytoplasm stained light lilac with small pink (eosinophilic, acidophilic) inclusions. Unstained areas of different size and number occurred in both forms (Fig. 3A, cf. PL(lr) and PL(SC)). Neutral red-positive cytoplasmic inclusions, which indicate lysosomes, were present in both spread and round hemocytes of the plasmatocyte type (Fig. 3F).

Spread plasmatocytes were the most abundant cell morphotype with a differential hemocyte count (DHC) of mean ± SD 86.89% ± 7.02% (Giemsa-stained monolayers; n = 3303 cells from n = 6 animals observed) (Fig. 4). They covered large areas on the glass substratum, since these cells flatten and project lamellipodia- and/or filopodia-like protrusions. These hemocytes spread evenly, resulting in a fried egg morphology, or spread unevenly, forming various shapes, including polarized (extending primarily to one direction) (Fig. 3A) and star or “sea urchin” shape (projecting numerous filopodia into each direction, e.g., Fig. 6A). The mean diameter measured 27.5 μm, but due to unsymmetrically spreading and the variability of shapes diameter between 12.5 and 38.3 μm were observed. The nucleus was either round, slightly oval or sometimes irregularly
shaped, with a mean diameter of 10.4 μm (minimum: 7.5 μm, maximum: 13.8 μm). Owing to the flattened shape, the cytoplasm area was high, with a mean N:C of 0.18 (minimum: 0.09, maximum: 0.28).

Round plasmatocytes had an even edge (without cytoplasmic protrusions), appeared circular or slightly oval, and contained no crystalline inclusions (PL(lr) in Fig. 3A). Seldom, these cells lacked the unstained vacuole-like areas after Giemsa-staining. The mean cell diameter measured 15.8 μm (minimum: 10.6 μm, maximum: 26.0 μm). Nuclei were mostly round, but also slightly oval or irregularly shaped nuclei occurred. The mean nucleus diameter was 8.2 μm (minimum: 6.2 μm, maximum: 11.5 μm). The cytoplasm area is large, with a mean N:C of 0.40 (minimum: 0.22, maximum: 0.58). With a DHC of mean ± SD 10.00% ± 7.07%, round plasmatocytes were the second most abundant morph observed (Fig. 4).

Besides the round plasmatocytes we found small round cells with similar staining properties. Some of these projected protrusions, while others did not. They lacked the characteristics of prohemocytes and crystal cells. Since in D. melanogaster larvae 90%–95% of the circulating hemocytes represent plasmatocytes (Rizki, 1957), we compared the blood picture of H. illucens with that of D. melanogaster. In naive D. melanogaster L3 larvae Giemsa-stained plasmatocytes occurred in smaller and larger forms (data not shown). This reflects strikingly the situation in H. illucens. Therefore, we integrated these morphs, which appeared in very low numbers (mean ± SD 0.62% ± 0.86% small round cells without protrusions; mean ± SD 0.77% ± 0.90% small round cells with protrusions; Fig. 4), to the plasmatocyte type in H. illucens.

Mitosis occurred rarely, and exclusively in round or spread plasmatocytes (Fig. 3D, E, G'). Hemocytes from n = 7 animals with at least n = 500 hemocytes per animal examined contained between 0% and 0.64% anti-PHH3 positive cells, mean ± SD 0.33% ± 0.25% (total hemocytes examined n = 8752; see Fig. 3G, G' for representative anti-PHH3-stained plasmatocytes).

The putative prohemocytes are round to oval shaped cells that lack protrusions, with a mean diameter of 11.9 μm (minimum: 7.1 μm, maximum: 15.8 μm) (Fig. 3B). Within the cells, we observed round or elongated nuclei with a mean diameter of 7.7 μm (minimum: 4.4 μm, maximum: 10.5 μm) (Table 1). The ratio of N:C within the cytoplasm was low, with a mean of 1.2 (minimum: 0.52, maximum: 2.19). Giemsa-stained prohemocytes often exhibited an intense blue to purple stained cytoplasm. These cells can also be reliably recognized both in nucleus and cell membrane or F-actin stained preparations (data not shown). The prohemocyte abundance was low, with a DHC of mean ± SD 0.73% ± 0.17% (Giemsa-stained monolayers; n = 3303 cells from n = 6 animals observed) (Fig. 4).

Crystal cells are relatively large, round to oval cells with a mean diameter of approximately 18.3 μm (minimum: 13.6 μm, maximum: 27.1 μm) and eccentric, round nuclei with a mean diameter of 7.7 μm (minimum: 5.7 μm, maximum: 10.1 μm). Fixed as well as living crystal cells were easily distinguished by their crystalline inclusions, which are eponymous for this hemocyte type (cf. crystal cell-like hemocytes in Zdybicka-Barabas et al., 2016). Crystalline inclusions measured between 2.4 and 14.6 μm in length (mean length 6.3 μm) and between 0.5 and 1.4 μm in width (mean width 0.9 μm). The mean N:C was 0.26 (minimum: 0.13, maximum: 0.53). The cytoplasm of Giemsa-stained crystal cells was darker blue stained than in other large round cells, the crystals remained unstained (Fig. 3A, C). Presence of (pro-)phenoloxidase ((Pro-)PO) was determined on acetone...
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**Fig. 3** The blood picture of *H. illucens* larvae. (A)–(E) Giemsa-stained fixed larval hemocytes. (F) Vital cell lysosome staining with neutral red. (A) The flattened cell type with uneven edges (spread plasmatocyte, PL(SC)) and round plasmatocytes (PL(lr)) are similar in their staining properties, that is, purple cytoplasm, pink cytoplasmic inclusions (eosinophilic granules, empty triangles) and unstained areas. (B) Prohemocytes (ProHC) are mostly small cells, with sparse cytoplasm and a comparable large nucleus (solid black arrow), and blue cytoplasm (see also ProHC marked by solid black arrow in D). (C) Crystal cells (CC) contain unstained crystalline intracellular structures (cs) and a blue cytoplasm. Rarely, CC contain two nuclei (nu in C). (D), (E) Round hemocytes rarely exhibit mitotic figures (white solid arrows) indicating hemocyte division in circulation (D, anaphase; E, prophase). (F) Both round hemocytes (PL(lr)) and spread hemocytes (PL(SC)) contain neutral red-positive compartments (lysosomes, in red), concentrated around the nucleus. (G), (G') Hemocytes labeled with mitosis marker anti-phosphohistone H3 (Ser 10) antibody (anti-PHH3; magenta) and nuclei stained with DAPI (blue). Spread plasmatocytes (PL(SC)) positive for anti-PHH3 are marked by solid white arrows in G'. Scale: A–F in same magnification (scale below E equals 20 μm); G, G' scales at lower right side equal 20 μm.

fixed hemocyte preparations by adding DAB as substrate together with hydrogen peroxide and the peroxidase inhibitor sodium azide. On these slides, only a few round cells without protrusions contained dark brown stained crystals, and light brown cytoplasm (Fig. 5). Crystal cells were consequently the only hemocyte type tested positive for (Pro-)PO. Peroxidase activity has been ruled out to be responsible for staining any hemocyte type by the use of specific inhibitors (Fig. 5C). The abundance of crystal cells was low with a DHC of mean ± SD 0.99% ± 0.74% (Giemsa-stained monolayers; \( n = 3303 \) cells from \( n = 6 \) animals).

**Screening for additional hemocyte markers**

With the morphological classification of three hemocyte types, the demand for more specific determination methods arose. We screened for reactivity of *H. illucens* hemocytes with a set of lectins and antibodies known to bind epitopes in other insect species (summarized in Tables S1 and S2).

Of all antibodies tested, only the anti-*D. melanogaster* Papilin antibody turned out to bind to all *H. illucens* hemocytes, most likely at the cell surface (Fig. 6A). Anti-*B. mori* proPO immune serum, anti-horseradish
Differential hemocyte count of Giemsa-stained hemocytes. Hemocytes were classified by their appearance as spreading cells (SC), small round plasmatocytes with protrusions (sr), small round plasmatocytes without protrusions (sr), large round plasmatocytes without protrusions (lr), crystal cells containing crystalline inclusions (CC), and prohemocytes with a high nuclear-to-cytoplasm ratio (ProHC). SC and lr most likely belong to one hemocyte type (plasmatocytes); srP and sr are most likely smaller variants of the SC and lr. The morphs within the dashed brackets are thus summarized as plasmatocytes (see main text for explanation). Insert depicts the hemocyte types with lower abundance (i.e., except SC) in magnified view. *Count of hemocytes excluding aggregates of more than three cells. Black dots denote outliers, grey and black dots mark each observation. Boxes indicate upper and lower quartiles, whiskers indicate 1.5-fold interquartile distance. Black horizontal bars depict median values.

Eight of the 12 lectins that were screened for reactivity labeled hemocytes (Table S2). Peanut agglutinin (PNA), Helix pomatia agglutinin (HPA), Maclura pomifera agglutinin (MPA), and wheat germ agglutinin (WGA) labeled all hemocytes present on the slides, and no obvious differential labeling pattern, which may aid in distinguishing hemocyte types was observed (Fig. 6, Table S1). Two lectins (PNA and HPA) bound to granular structures, which most likely represent intracellular granules in each hemocyte (Fig. 6A, D; Table S1). Two lectins (MPA and WGA) bound most likely to the cell surface of each hemocyte (Fig. 6B, C, Table S1).

The lectins LEA, PSA, and SBA were also screened for reactivity in a limited number of samples (n = 2 hemocyte monolayers with and without detergent each) and must therefore be investigated in detail in the future. LCA labeled each hemocyte with a patchy pattern, most likely granular inclusions (Table S1), in a pattern similar to MPA and WGA. The lectins LEA and PSA differed from the labeling pattern of the other hemocyte binding lectins in so far as they labeled round cells, most likely prohemocytes, intensely at the cell membrane while each other hemocyte types contained lectin-labeled granules in the cytoplasm (Table S1).

**Phagocytosis of microorganisms by hemocytes**

The ability of hemocytes to take up microorganisms was tested by injection of fluorescence-labeled dead bacteria or yeasts and subsequent determination of the presence of intracellular fluorescence signal in hemocytes at distinct time points. The hemocytes were additionally stained with Phalloidin to distinguish between microorganisms that adhere at the cell surface and intracellular located microorganisms. In vivo phagocytosis of dead FITC-labeled bacteria (E. coli BL21 [DE3], B. subtilis) appeared to be rapid, with hemocytes containing bacterial particles 15 min after injection (the shortest incubation time tested, Table 2). Uptake of larger eukaryotic microorganisms seemed to be slower; 60 min after injection, no hemocytes contained dead, FITC-labeled Saccharomyces boulardii bioparticles (Table 2). After 150 min, however, numerous hemocytes incorporated the yeast particles. In both bacteria- and yeast-injected animals, no fluorescence has been observed in the hemocytes after 24 h, which indicates a complete clearance and intracellular degradation of the foreign particles at this time point (Table 2).

The percentage of hemocytes containing microorganisms was determined by fluorescence microscopy. As a proof of principle, and to ensure that colocalization of fluorescent bioparticles and hemocytes is the result of true phagocytosis, we conducted additional in vivo phagocytosis experiments and analyzed the microbe uptake of hemocytes in vivo. For this, we injected FITC-labeled dead B. subtilis and compared the pattern of...
fluorescent particles in the hemocytes after adding trypan blue to quench fluorescence of nonphagocytized bacteria with the pattern of nonquenched samples. In both cases, the bacteria were predominantly localized in the perinuclear cytoplasm (Fig. S2). Further, we labeled dead bacteria (E. coli K12 D31) and dead yeasts (S. boulardii), with the pH-sensitive fluorescent dye pHrodo, and tested for phagocytosis. When phagosomes containing phagocytized particles fused with the acidic lysosomes, the fluorescent signal becomes stronger, indicating true phagocytosis, as shown in Fig. S3. Both experimental setups confirmed that hemocytes truly phagocytize microbes.

For the quantification of phagocytic events, the hemocytes were fixed and counter-stained with Phalloidin to discriminate between intracellular microorganisms and microorganisms adhering at the hemocyte membrane. Strikingly, the vast majority of circulating hemocytes contained E. coli or B. subtilis. The larger yeast cells, however, were found in a smaller percentage of circulating hemocytes (Fig. 7A). Plasmatocytes, which we subdivided into four morphologically distinct subtypes (see Giemsa-staining, cytometry and (pro-)phenoloxidase activity of hemocytes), were the only hemocytes containing intracellular bacteria or yeasts, while prohemocytes never contained microorganisms (Fig. 8). No crystal cells were unambiguously identified in these preparations.

These results were compared with live hemocytes 30 min after injection of FITC B. subtilis. In this experiment, the hemocytes form each animal were divided before either adding trypan blue (0.2% w/v) final concentration (or PBS. In both cases, internalized bacteria were easily distinguishable from occasionally occurring non-phagocytized bacteria. Bacteria within the hemocytes aggregated in the cytoplasm, often forming a circle-like shape around the nucleus, which appear brighter than nonphagocytized cells after adding trypan blue. Localization of microorganisms within vicinity to the nucleus, that is, in the cytoplasm rich circumnuclear region of the hemocytes, was therefore a diagnostic feature to identify phagocytized bacteria. Live hemocytes containing bacteria were round or spread plasmatocytes, and the overall pattern resembled that observed in the fixed and counter-stained preparations.

When freshly harvested hemocytes were brought into contact with live GFP expressing B. subtilis, the bacteria were immediately immobilized upon contact to the hemocyte surface. In response to bacteria contact, the hemocytes projected fine protrusions (Movie S2) or lamellipodia toward the adhered bacteria (Fig. 8; Movies S1 and S2). Lamellipodia that have been formed prior to contact to bacteria also moved relatively fast in order to engulf the bacteria (Fig. 8; Movies S1–S3). The trapped bacteria were engulfed within few minutes, indicating a rapid activity even under in vitro conditions. We also tested the ability of hemocytes to engulf dead FITC-labeled S. boulardii. The hemocytes, however, did not actively contact or engulf the yeast particles in vitro within 3 h of observation (data not shown).
Fig. 6 Antibody and lectin labeling patterns of hemocytes. Nuclei stained with DAPI (blue). Insets demarcate cells from other slide positions. (A) Double labeling with anti-D. melanogaster Papilin immune serum (PAP, magenta) and peanut agglutinin (PNA, green; carbohydrate specificity: Galβ1-3GalNAc). Anti-PAP labels all hemocytes, most likely on their cell surface. PNA labels granular cell components, most likely intracellular inclusions. In spread plasmatocytes, these concentrate around the nucleus (e.g., cell in the upper left box). A round cell without pseudopodia (most likely a round plasmatocyte, PL(r)) is depicted in the lower right corner. (B) The Maclura pomifera agglutinin (MPA, magenta; carbohydrate specificity: Gal[β1–3]GalNAc) labels all hemocytes most likely on the cell surface. The cells were permeabilized with Triton X-100 prior to labeling. The cells shown here represent mostly spread plasmatocytes and smaller round plasmatocytes. (C) Wheat germ agglutinin (WGA, magenta; carbohydrate specificity: GlcNAc) labels the cell surface of all hemocytes. The hemocytes were treated with Triton X-100 before labeling. Each cell shown in this picture belongs to the plasmatocyte type. (D) Helix pomatia agglutinin (HPA, magenta; carbohydrate specificity: GalNAc) labels granular inclusions of round and spread cells. Each cell shown was classified as plasmatocyte, with some uncertainty for the cell shown in the inset in the lower left, which may also be a prohemocyte. The upper insets on the right side depicts the HPA-signal merged with DIC, the lower depicts the HPA-signal merged with the DAPI-signal. Note that the HPA-positive granules localize in the central cytoplasm around the nucleus rather than in the pseudopodia. Scale bar in A (25 μm) is valid for all pictures.

Discussion

Recently, the humoral immune response of the emerging dipteran model H. illucens gained interest by researchers worldwide (e.g., Müller et al., 2017; Vogel et al., 2018; Moretta et al., 2020; Xia et al., 2021). In order to get a comprehensive understanding on the immune response of H. illucens, we elucidated the cellular composition of the hemolymph and cell mediated antimicrobial activity. Hence, our aim was (1) to characterize the circulating hemocytes, and (2) to examine the phagocytosis competence of the hemocytes in H. illucens larvae.
Table 2  Determination of ingested fluorescent microorganisms in hemocytes at distinct time points after injection.

| Microorganism     | t 15 min | t 30 min | t 60 min | t 150 min | t 24 h |
|-------------------|----------|----------|----------|-----------|--------|
| E. coli BL21 (DE3)| +++      | +        | +        | +         | −      |
| B. subtilis       | +++      | +        | +        | +         | −      |
| S. boulardii      | ND       | −        | −        | −         | +      |

At least $n = 6$ larvae per treatment; +, microorganisms present in hemocytes; −, no microorganisms present in hemocytes; ND, not determined.

### Total hemocyte counts

Determination of the total hemocyte number in insects is known to be dependent on several methodological factors, including site and method of hemolymph collection and pretreatment of the animals (e.g., Tauber & Yeager, 1935, 1936; Jones & Tauber, 1951; Jones, 1967; Castillo et al., 2006; reviewed in Salt, 1970; Siddiqui & Al-Khalifa, 2014). Therefore, we first established a method to collect hemocytes from *H. illucens* larvae by testing several methods including withdrawal of hemolymph with a syringe, cutting incisions at different body parts and temperature treatment prior to collection. An incision set at the dorsal side of the posterior abdomen led to the highest amounts of hemolymph (approximately 10–40 μL hemolymph per animal) and lowest number of contaminations by gut or Malpighian tubule content or fat body fragments (data not shown).

We analyzed the effects of different temperature treatments on hemolymph yield and hemocyte density. Cooled larvae gave insufficient amounts of hemolymph, with very low hemocyte concentrations (data not shown). Heat-treatment of the larvae prior to hemolymph collection turned out to be superior over using larvae at room temperature or chilled larvae for the determination of the hemocyte concentration. The number of hemocytes per volume collected from heat-fixed larvae was approximately three times higher than in untreated larvae (Fig. 1A). This effect is also known from several other species (e.g., Jones, 1954; Bahadur & Pathak, 1971; summarized in Siddiqui & Al-Khalifa, 2014), and could be explained by (1) reduced adhesion during the sampling process, (2) by mobilization of hemocytes from (transient) sessile niches, or (3) a combination of these factors. Heat-fixation has the additional beneficial effects that hemolymph coagulation does not occur and that fixed hemocytes do not adhere at the wound site, which again could impair the reliability of THC estimations (cf. e.g., Matsumotu & Sakurai, 1956; Jones, 1962; Siddiqui & Al-Khalifa, 2014). Salt (1970) summarized the published numbers of circulating insect hemocytes for different species, and lists THCs ranging from less than one hundred to more than two million cells per μL. Even within a single species, the immune or developmental status may tremendously influence the number of harvestable circulating hemocytes (e.g., Tauber & Yeager, 1935, 1936; Webley, 1951; summarized in Jones, 1962; Gupta, 1979; Siddiqui & Al-Khalifa, 2014). Here, we determined for *H. illucens* larvae approximately 1100 cells/μL, which is a relatively low THC compared to THCs published for other holometabolous insects (see e.g., Tauber & Yeager, 1936; Salt, 1970). Examples of THCs published for brachyceran Diptera species are shown in Table 3. As far as we know only one examination of the THC of a Stratomyidae species, *Microchrysa* sp., is published (Gött et al., 1977). Its hemolymph contains 1000 cells/μL, albeit the method of collection and determination is unknown. Hence, even within the dipteran clade, the hemocyte density of *H. illucens* is relatively low.

To exclude bias by possible differences in the number of circulating hemocytes depending on body weight of the sampled animals, we analyzed the putative correlation between body weight and THC. The THC of the sampled larvae of the last feeding stage did not significantly correlate with the body weight. Only a weak positive tendency was observed between body weight and THC (Fig. 1B). Therefore, we conclude that intrastage variations of the hemocyte concentration represent the naturally occurring variation between individuals, and that our test method produces reliable values allowing comparison of THCs between different treatments. The method has been optimized for the last feeding instar, and we cannot exclude that between distinct developmental stages, and especially during molting, the THC changes.

Wounding affects the number of circulating hemocytes

The hemolymph of naive *H. illucens* larvae contains approximately 1100 hemocytes per μL. In the case of
Fig. 7 In vivo phagocytosis of dead microorganisms by hemocytes. Plasmatocytes contain ingested FITC-labeled (green) yeasts (A, S. boulardii), Gram-positive bacteria (B, B. subtilis) or Gram-negative bacteria (C, E. coli). The bar plots depict the corresponding percentage of hemocyte types containing microorganisms, with green indicating number of hemocytes containing microorganisms and blue indicating hemocytes without microorganisms. Mean of $n = 5$ hemocyte monolayers, each prepared from $n = 1$ animal injected. Hemocytes were harvested 150 min after injection (S. boulardii) or 30 min after injection (B. subtilis, E. coli). Plasmatocytes (PL) were subdivided into their morphologically distinct forms (lr, large round; sr, small round without protrusions; srP, small round with protrusions; SC, spreading cells) (see also main text for explanation). Prohemocytes (ProHC) never contained ingested microorganisms. Crystal cells were not observed and therefore excluded. Abbreviations: *total HC, whole hemocyte population excluding aggregates of more than three cells; SC, spread plasmatocytes; srP, small round cells with protrusions; sr, small round cells without protrusions; lr, large round cells without protrusions. Scale bar equals 20 $\mu$m.

Wounding or other immunological challenges the total number of circulating hemocytes may alter, as has been demonstrated in other insects (e.g., Horohov & Dunne, 1982; Christensen et al., 1989; Charles & Killian, 2015). The hemocyte concentration of H. illucens larvae was significantly reduced in response to wounding, most likely because hemocytes become adherent and are involved in wound closure, comparable to the hemocyte reaction described from other species. Early hemocyte response to wounding involves aggregation and adhesion to the wound site (e.g., Rowley & Ratcliffe, 1978; Babcock et al., 2008; Thuma et al., 2018) followed by formation of a preliminary wound closure (Rowley & Ratcliffe, 1978; reviewed in Wittig, 1962; Lavine & Strand, 2002). Consequently the number of hemocytes in circulation decreases while they are recruited to the wound site or other immunological threats. Short time depletion observed 1 and 2 h after wounding in H. illucens larvae is likely caused by hemocyte recruitment to the wound site, probably together with hemocyte immobilization by clotting reaction. Since 24 h after wounding the number of hemocytes nearly reaches the level of naive animals, the hemocyte population may be replenished by hematopoiesis, or hemocytes not used up by the wounding process enter again circulation. Circulating hemocytes may contribute to hemocyte replenishment by mitotic division (see mitotic figures in hemocytes of naive larvae; Fig. 3D, E), as it is the case in other insect
Fig. 8 In vitro uptake of live B. subtilis by hemocytes. Still images taken from Supplementary Movies S3 (A1–A5) and S2 (B1–B5). A1–A5, low bacteria abundance; B1–B5, high bacterial abundance. Merge of GFP (green) and bright-field. A1–A5: Directed movement of a lamellipodium with contact to a bacterium. Hemocytes cultured approximately 8 min before \(2 \times 10^6\) B. subtilis GFP/mL were added. First picture taken approximately 1 min after adding bacteria. The spread hemocyte transports an attached bacterium toward the center of the cell by lamellipodia movement and bending (solid black arrowheads). B1–B5: Pseudopod projection and engulfment of numerous bacteria. Hemocytes cultured approximately 6 min before \(2 \times 10^7\) B. subtilis GFP/mL were added. First picture taken approximately 1 min after adding bacteria. Note the projection of pseudopodia (solid black arrowheads) and subsequent bending and roll-up movement of the lamellipodia, finally engulfing the bacteria. The bent arrow in B5 depicts the movement of the lamellipodium in lateral view. Scale bars equal 20 \(\mu\)m.

### Table 3 Published total hemocyte counts of different brachyceran Diptera species.

| Family       | Species            | Approximately THC (cells/\(\mu\)L) | Method            | Reference                  |
|--------------|--------------------|-----------------------------------|-------------------|----------------------------|
| Tephritidae  | Anastrepha obliqua | 210 ± 144.5\(^a\)                | Unfixed           | Silva et al. (2002)        |
| Drosophilida | Drosophila melanogaster | 3078 ± 1706\(^e\)            | Unfixed           | Brehélin, 1982             |
| Drosophilida | Drosophila yakuba  | 3395 ± 1397\(^d\)                | Unfixed           | Brehélin (1982)            |
| Sarcophagida | Sarcophaga ruficornis | 5000 to 10 000\(^b\)          | Cold-anaesthetized | Pal and Kumar (2014)       |
| Muscidae     | Musca domestica    | 2000 to 5000\(^b\)               | Cold-anaesthetized | Pal and Kumar (2014)       |
| Calliphorida | Chrysomya megacephala | 2000 to 11 000\(^b\)          | Cold-anaesthetized | Pal and Kumar (2014)       |
| Stratiomyidae| Microchrysa sp.    | 1000\(^c\)                       | Cold-anaesthetized | Götz et al. (1977)         |
| Stratiomyidae| Hermetia illucens  | 334 ± 212\(^a\)                   | Unfixed           | This paper                 |
| Stratiomyidae| Hermetia illucens  | 1138 ± 588\(^b\)                  | Heat-fixed        | This paper                 |

\(^a\)Mean ± SD.

\(^b\)Estimated values retrieved from published diagram.

\(^c\)Unknown method.

(e.g., Jones, 1962; King & Hillyer, 2013; Duressa et al., 2015).

We did not analyze the changes in the circulating hemocyte concentration in response to microbe injection since injection of the microbes is either accompanied by injection of fluid, thereby diluting the hemolymph (injection of microbes in a carrier fluid) or the microbes are introduced by a contaminated needle, which would likely result in a
large deviation of bacterial load between the animals. It is likely, however, that the initial decrease of circulating hemocytes after microbe injection will be even higher than the decrease caused by wounding alone due to (1) recruitment of the cells to the wounding site and (2) additional immobilization of hemocytes caused by the antibacterial response (aggregation and nodulation).

Identification of circulating hemocyte types

Here, we present a first analysis of the hemocyte types in *H. illucens* according to histological, cytometric, and functional features. With these analyses, we gained a first insight into the cellular composition of the larval hemolymph.

A difficult task was to decide which nomenclature for hemocytes to follow. In the case of prohemocytes and crystal cells, despite their low abundance, the situation was clear in *H. illucens*. Prohemocytes, defined as small round cells with a high nucleus-to-cytoplasm-ratio, were easily distinguishable; crystal cells contained refracting crystals in their cytoplasm. On the other hand, distinguishing plasmatocytes from granular cells is often confusing (summarized in Ribeiro & Brehélin, 2006). Generally, it is assumed that granular cells are phagocytic cells, which may spread symmetrically, whereas plasmatocytes may or may not be involved in phagocytosis, spread irregularly and are the cells that perform encapsulation (summarized in Ribeiro & Brehélin, 2006). However, these characteristics are often weak or even interchangeable between the types (see e.g., encapsulation by granular cells: Pech & Strand, 1996; symmetrical spreading of plasmatocytes and asymmetrical spreading of granular cells: own observations in *M. sexta*; phagocytic activity of plasmatocytes and even hyperphagocytosis: Ratcliffe & Rowley, 1974; Dean et al., 2004, own observations in *M. sexta*).

Spreading phagocytes in the mosquito are for instance termed granular cells and are not called plasmatocytes, even when the plasmatocyte-typic asymmetrical spreading occurs (Castillo et al., 2006). It is therefore difficult to determine differences between granular cells and plasmatocytes as long as specific markers are lacking, and nomenclature of cells that are able to spread and to internalize foreign material depends largely on the author and on the weighting of specific features rather than following a—hypothetical—strictly regulated nomenclature (cf. e.g., discussion of mosquito hemocyte types in Castillo et al., 2006). We identified the most abundant hemocyte population in *H. illucens* to resemble either granular cells (containing eosinophilic /acidophilic granules typical for granular cells, cf. Elefltherianos et al., 2021) or plasma-

tocytes (since asymmetrical spreading was observed regularly). Following the *D. melanogaster* hemocyte classification (Rizki & Rizki, 1980; Shrestha & Gateff, 1982; Williams, 2007), we termed these cells plasmatocytes.

We tend toward a conservative hemocyte classification scheme rather than to an overinterpretation of minor differences. Therefore, three main hemocyte types were identified in *H. illucens* larvae in our study: plasmatocytes, crystal cells, and prohemocytes.

The plasmatocytes occurred in a variety of shapes and sizes. They appeared as spread or unspread cells, and a subset seemed to be smaller. Their Giemsa-staining properties, however, were indistinguishable, and the nucleus size and shape as well as the presence of vacuole-like structures in Giemsa-stained preparations were also similar in these cells. Additionally, small as well as large plasmatocytes of microorganism-injected animals contained microorganisms, indicating phagocytosis competence. When live hemocytes were observed, granularity of the cytoplasm appeared to alter with progressing culture time, as it was also the case for the shape (flattening, spreading, and protrusion formation).

Prohemocytes are in general described by morphological rather than any other criteria. These cells appear to possess only sparse cytoplasm, are mostly round in shape, and have a high nuclear-to-cytoplasm-ratio (Gupta, 1979; Ribeiro & Brehélin, 2006). Regarding the nuclear-to-cytoplasm-ratio and staining properties of Giemsa-stained hemocytes, prohemocyte candidates were easily distinguished from each other hemocyte type: the cytoplasm appears often dark blue and covers only a small area compared to the nucleus size, the cells are very minute compared to each other hemocyte type. They contribute less than 1% of the circulating cells.

The (pro-)phenoloxidase ((Pro-)PO) containing hemocytes in insects are usually called oenocytoids, or crystal cells if ProPO is stored in crystalline inclusions. The difference between oenocytoids and crystal cells is therefore superficial (reviewed in Ribeiro & Brehélin, 2006). The *H. illucens* crystal cells are easy to distinguish from all other cell types by their prominent rod shaped crystalline inclusions (Zdybicka-Barabas et al., 2016, this paper). The crystalline inclusions are similar in shape to the crystals found in crystal cells of *D. melanogaster*. Giemsa-stained crystal cells exhibited a round nucleus, dark-stained cytoplasm, and nonstained crystals. When acetone fixed hemocytes were incubated with DAB and hydrogen peroxide, the crystal cells became brown. Strikingly, when the PO-inhibitor PTU was added, all cells remained unstained, while adding the peroxidase inhibitor sodium azide did not alter the staining pattern. Therefore, we conclude that, similar as for *Manduca sexta* PO
(von Bredow et al., 2020; Holthusen & Trenczek, unpublished) or *Mytilus edulis* PO (Renwrantz et al., 1996), one or more putative phenoloxidase enzymes produces a precipitate from the substrate DAB. Thus, one or more PO(s) are present in the crystal cells. The strong staining of the crystals themselves indicates that (Pro-)PO is stored in the crystals. Comparable to ProPO containing hemocytes of other insect species, the crystal cells of *H. illucens* make up only a small portion of the circulating hemocytes (approximately 1%), and are assumed to release the (Pro-)PO(s) in response to an immune stimulus.

A recent study by Cattenoz et al. (2020) revealed for example fourteen hemocyte subtypes by single cell transcriptome analyses in *D. melanogaster*. Similarly, Tattikota et al. (2020) identified 16 hemocyte subtypes (alias clusters) and one circulating nonhemocyte cell type by single cell RNA-seq in parasitized *D. melanogaster*. Markers that allow discrimination of specific hemocyte types based on the localization of the labeling. The carbohydrate specificity of the lectins differs (LCA: Man, Glc, Fucose-linked α(1–6) to core GlcNAc of N-linked Glycopeptides; LEA: GlcNAc, (GlcNAcb1–4)n, (Galb1–4GlcNAc)n (polylactosamine); HPA: GalNAc, GalNAcα1–3GalNAc (Forssman-antigen), MPA: Gal, Gal-α/GalNAcα-1/Core1; GalNAcα-Ser (Tn-antigen); PNA: Gal, Galβ1–3GalNAc of N-glycans and glycolipids; PSA: Man, Glc, High-Man-type N-glycans, biantennary N-glycans containing core α1-6Fuc; SBA: GalNAc, GalNAcα1–3Gal of O-linked Glycopeptides; WGA: GlcNAc, Sia, GlcNAc(b1–4GlcNAc)n; according to Kobayashi et al., 2014). Hence, we conclude that a variety of glycoconjugates is present in granules and on the cell surface of *H. illucens* hemocytes. The HPA- and PNA-positive granules are probably partly identical with the granules observed in light microscopy of live hemocytes (cf. Figs. 8 and S1). In the nematoceran Diptera species *Anopheles gambiae* and *Aedes aegypti* HPA, PNA, and WGA labeled each circulating hemocyte type (HPA and WGA in *A. aegypti*: Hillyer & Christensen, 2002; HPA, WGA, and PNA in *A. aegypti* and *An. gambiae*: Castillo et al., 2006), and Kwon et al. (2021) utilized WGA labeling for hemocyte separation in *An. gambiae*. Interestingly, PNA also labels the granules in the cytoplasm of *M. sexta* granular cells (Nardi, 2004; von Bredow et al., 2020) and additionally of oenocytoids (von Bredow et al., 2021) but not of plasmatocytes. The differential binding patterns of the lectins LEA, PSA, and SBA, however, indicate that differences in the glycosylation patterns may be used to distinguish certain hemocyte types in future works. SBA, for example, has been shown to preferentially label phagocytizing hemocytes in the fly *Calliphora vomitoria* (McKenzie & Preston, 1992).
Our data may provide useful tools for future studies on *H. illucens* hemocytes. However, this is a first screen for hemocyte binding lectins, and glycosylation patterns may alter depending on factors such as developmental stages (Varki et al., 2017). Therefore, future experiments should include, for example, inhibition with specific sugars, analysis of labeling patterns in different developmental stages (larva, pupa, imago), and immunological backgrounds (infected vs. naive).

Mitosis occurred only very seldom in *H. illucens* hemocytes (Fig. 3D–G, G′). Interestingly, only hemocytes we designated as plasmatocytes showed signs of mitosis (disassembled nuclear envelope, orientation of chromosomes or cytokinesis, and phosphorylation of Ser10 of histone H3). When the individual chromosomes were visible, the hemocytes contained 7 pairs of chromosomes, which is concordant with 1 n = 7 chromosomes described by Boyes (1973) for this species. Antibody labeling with the mitosis marker anti-phosphohistone H3 (Ser10) confirmed that only a low number of the circulating round or spread plasmatocytes from naive animals are dividing (mean ± SD 0.33% ± 0.25%).

Another seldom observed phenomenon was that crystal cells may contain two nuclei (Fig. 3C), probably caused by incomplete mitosis, since no crystal cell has been observed going through cytokinesis.

The embryonic and larval hematopoiesis of *H. illucens* is yet not examined. In future studies, both the hematopoietic potential of circulating hemocytes (with special emphasis to prohemocytes and plasmatocytes) as well as of yet not identified sessile hemocytes should be investigated. It is highly likely that a larval hematopoietic organ comparable to the lymph gland in *D. melanogaster* larvae or similar hematopoietic organs exists and hemocytes are formed not only by mitosis of circulating hemocytes but also formed and released by sessile hemocytes or specialized tissues. The question whether hemocytes are formed solely by mitosis and differentiation (or trans-differentiation) in circulation or additionally by specialized organs should be addressed in future studies.

**Phagocytosis and bacteria elimination by hemocytes**

Phagocytosis is a potent mechanism to eliminate potential pathogens from the hemolymph, mainly mediated by the circulating immune cells (Ratcliffe & Rowley, 1974; Lavine & Strand, 2002; Haine et al., 2008; Strand, 2008). Due to its rapid mode of action, phagocytosis belongs to the fast immune response. Its outstanding role in the removal of invading pathogens has been shown by Haine et al. (2008), who described that the vast majority of bacteria injected in *Tenebrio molitor* is incapacitated before the AMP-mediated humoral antimicrobial activity increases.

The fast uptake of injected bacteria by *H. illucens* hemocytes within 15 min after injection, as demonstrated in the present paper, underlines that phagocytosis is also part of the first and rapid immune response in *H. illucens*. Similarly, when freshly recovered hemocytes came in contact with live *B. subtilis* in vitro, it took only a few minutes from the first contact to ingestion or at least engulfment by the hemocyte (Fig. 8, Movies S1–S3), which is in a similar time frame like the rapid phagocytosis described for *Aedes aegypti* hemocytes (Hillyer et al., 2003). This process involved immobilization of the bacterium by attachment to the hemocyte surface, and expansion of cytoplasmic protrusions or movement of existing lamellipodia toward the bacterium (Fig. 8, Movies S1–S3). The lamellipodia participating in the bacteria uptake regularly lost contact to the glass surface of the culture chamber (Fig. 8A3), and rolled up in order to transport the bacterium to the central hemocyte portion (Fig. 8A4–A5), which houses the nucleus and a portion of granulated cytoplasm. If this process in vitro finally led to a true phagocytosis, or stops after the bacteria are engulfed by the protrusions remains open, but the process shown in Movie S3 and Figure 8A strongly indicates a true phagocytosis process of a single bacterium. In order to clarify whether a true phagocytosis takes place, we conducted in vivo phagocytosis experiments with both pHrodo-labeled *E. coli* or *S. boulardii*. In both cases, the microbes internalized by hemocytes exhibited a strong fluorescence, indicating that the microbes were enclosed by phagosomes that fused with acidic lysosomes (Fig. S3). Therefore, we conclude that particles colocalized with hemocytes are indeed phagocytized rather than attached to the cell surface. A comparison of the microbe distribution within the hemocytes between trypan blue quenched and untreated hemocytes after injection with FITC-labeled *B. subtilis* further supports this interpretation (Fig. S2).

However, uptake of larger bioparticles such as yeasts takes longer to initiate in vivo, but after 2.5 h also a large number of internalized yeasts was recorded. An in vitro approach to track phagocytosis of dead yeast was not successful (data not shown).

It turned out that, like in other insects, a defined hemocyte type—the plasmatocyte—is responsible for pathogen uptake. Within the plasmatocyte population, the majority was able to phagocytize injected microorganisms. The percentage of plasmatocytes containing microorganisms was much higher for bacteria than for the yeast. We hypothesize (1) that the size of the injected yeasts may impair the ability of smaller hemocytes to successfully phagocytize the large intruders (Fig. 8) and (2)
that the phagocytosis of yeasts depends on other soluble (opsonins) or cell membrane bound factors (phagocytosis receptors) than phagocytosis of bacteria (cf. e.g., TEPs, Stroschein-Stevenson et al., 2005; reviewed in Stuart & Ezekowitz, 2008).

We conclude that the plasmatocytes are both the professional phagocytes and the only phagocytosis competent hemocyte type, since crystal cells or prohemocytes have never been found to contain bacteria or yeasts. Recalling that plasmatocytes also make up the vast majority of all circulating hemocytes in *H. illucens*, it becomes clear that the cellular immune response is a potent mechanism to fight invading pathogens in this species.

In preliminary experiments, we observed that in vivo clearance of injected live *E. coli* is complete 16 h after injection (data not shown, in preparation). This indicates that *H. illucens* larvae are capable to efficiently clear invading microorganisms from circulation and is congruent with the observations presented in this paper that 24 h after injection no FITC-labeled dead microorganisms were observed in hemocytes. Clearance of viable bacteria from circulation is most likely caused by a combination of antimicrobial (humoral) activity and immobilization of the bacteria by, for example, phagocytosis, encapsulation, or nodulation. Besides phagocytosis by circulating hemocytes, we found evidence that sessile cells or tissues also play a role in particle clearance in *H. illucens*. Injected carbon particles (china ink) as well as injected FITC-labeled yeasts accumulated rapidly in sessile pericardial tissues (own observations, data not shown). The process of particle clearance has been assigned to nephrocytes or pericardial sessile hemocytes for orthopteran and dipteran insects (Cuénot, 1895; Nutting, 1951; Hoffmann, 1970; King & Hillyer, 2013; Sigle & Hillyer, 2016) and has recently been shown in a large study of 68 insect species to be a general trait of insects (Yan & Hillyer, 2020). It has also been proposed that phagocytic active hemocytes of the mosquito *Anopheles gambiae* preferentially aggregate at the ostia of the dorsal vessel (Sigle & Hillyer, 2016), indicating that a similar mechanism may lead to particle aggregation at pericardial sites in *H. illucens*.

**Concluding remarks and outlook**

The larval hemolymph of *H. illucens* contains at least three hemocyte types: prohemocytes, plasmatocytes, and crystal cells. Prohemocytes may resemble progenitor cells, crystal cells contain (pro-)phenoloxidase, and plasmatocytes are the most abundant type and perform phagocytosis. We are convinced that *H. illucens* is a promising model not only for commercially relevant research. With the fully sequenced genome and the possibility of genetic manipulation (Zhan et al., 2020), the enormous genetic variability (Ståhls et al., 2020), and its phylogenetic position within the brachyceran Diptera clade (Brammer & von Dohlen, 2007; Yeates et al., 2007) *H. illucens* is a promising model for comparative developmental and immunological research. Due to its enormous economical potential, breeding plants are built and maintained worldwide; therefore, the animals will be readily available, and in return the basic research on its biology is fundamental for optimization of breeding and biomass generation. Understanding immunity of *H. illucens* is crucial regarding the potential economic losses, which can be caused by pathogens or parasites. We suggest that future research should not only focus on humoral activity but also on understanding the cellular immunity, including hematopoiesis, hemocyte differentiation, the identification and generation of reliable markers, and the role and function of distinct hemocyte types in distinct immunological events.

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**Disclosure**

The authors declare that there are no competing interests.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Changes of hemocyte morphology in *vivo*.

**Fig. S2** *In vivo* phagocytosis of FITC-labeled *B. subtilis* by hemocytes of *H. illucens*.

**Fig. S3** *In vivo* phagocytosis of pH-sensitive fluorochrome-labeled microorganisms.

**Table S1** Screen for hemocyte binding lectins.

**Table S2** Screen for hemocyte binding antibodies, enzymatic activity, and vital stains.

**Movie. S1** *In vitro* bacteria engulfment by hemocyte lamellipodia.

**Movie. S2** *In vitro* bacteria immobilization by hemocyte filopodium and subsequent engulfment.

**Movie. S3** Transport of immobilized bacterium by hemocyte lamellipodium.