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RESEARCH REPORT

Distinguishing *Manduca sexta* haemocyte types by cytometric methods

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Abstract

Manduca sexta larvae possess haemocytes which are classified according to their appearance into five distinct types typical for Lepidoptera: plasmatocytes (PLs), granular cells (GRs), spherule cells (SPs), oenocytoids (OEs), and prohaemocytes (ProHCs). Some haemocytes do not fit morphologically into either class, i.e. sharing morphological criteria of two cell types, obscuring their classification. We characterised larval haemocytes of *M. sexta*, whose identity was confirmed by specific markers, based on morphometric and cytometric methods by light microscopy and fluorescence microscopy. For each haemocyte class, the size, the length-to-width-ratio, the nuclear morphology, the relative DNA-content and the nucleus-to-cytoplasm-ratio were measured. PLs and GRs occur in either round or spread form, the latter resulting in a high length-to-width-ratio. The shape of the nuclei also differed between haemocyte types, with almost round nuclei in GRs, ProHCs and OEs, and oval to irregularly formed nuclei in SPs and PLs. The nucleus-to-cytoplasm-ratio was shown to differ significantly between each type. PLs and OEs exhibited polyploidy, while granular cells, spherule cells and putative prohaemocytes were presumably 2n (G0/G1) or 4n (G2/M). Finally, a dichotomous guide allowing determination of the haemocyte types by means of light microscopy and DNA labelling was created.

Key Words: *Manduca sexta* larvae; haemocyte types; haemocyte morphometry; haemocyte cytometry; haemocyte ploidy; haemocyte identification key

Introduction

Free circulating cells in the insect haemocoel are called haemocytes. These cells are major players in immunity and homeostasis, conducting cellular immune responses such as phagocytosis and encapsulation (Horohov and Dunn, 1982; Wiegand *et al*., 2000; Lavine and Strand, 2002) and participate in extracellular matrix deposition and degradation (Nardi and Miklasz, 1989; Kusche-Gullberg *et al*. 1992; Bunt *et al*., 2010; Sánchez-Sánchez *et al*., 2017). Basically, the haemocyte types or classes are classified based on morphological characteristics (Jones, 1962; Price and Ratcliffe, 1974; Gupta, 1979; Horohov and Dunn, 1982). Functional analyses revealed specialised actions of distinct haemocyte types. The oenocytoids of lepidopteran insects for example synthesize and release prophenoloxidase, the key component of the melanisation reaction necessary to fight large pathogens (Iwama and Ashida, 1986; Jiang *et al*., 1997), plasmatocytes and granular cells phagocytose or encapsulate foreign bodies (Pech and Strand, 1996; Levin *et al*., 2005) and prohaemocytes are thought to divide and differentiate to form other haemocyte types (Yamashita and Iwabuchi, 2001) and can therefore be seen as either progenitor cells or stem cells with limited differentiation potential. By developing

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List of abbreviations:
β-1,3-glucan recognition protein 1, βGRP-1; 4',6-diamidino-2-phenylindole, DAPI; fluorescein isothiocyanate, FITC; granular cell, GR; length-to-width-ratio, L:W; nucleus, nuc.; nucleus-to-cytoplasm-ratio, N:C; oenocytoid, OE; plasmatocyte, PL; spread granular cell, sprGR; spread plasmatocyte sprPL; peanut agglutinin, PNA; PNA-positive cell membrane, PNAm+; prohaemocyte, ProHC; round plasmatocyte, rPL; spherule cell, SP; tetramethylrhodamine isothiocyanate, TRITC; Tris-buffered saline, TBS

Table 1 Marker molecules used for haemocyte determination

Primary antibody	Epitope and/or cell type specificity	Host / type	Ig type / subtype	Inventor / References / RRID	Dilution
mAb MS7	Granules in granular cells, unknown epitope	mouse mAb	IgG	Willot *et al.,* 1994 / Beetz et al. 2004, 2008, this paper	hybridoma supernatant
mAb MS13	β-Integrin expressed on *M. sexta* plasmatocytes and haematopoietic organs	mouse mAb	IgG2b	Willot *et al.,* 1994 / Wiegand *et al.,* 2000; Nardi *et al.*, 2003; Beetz *et al.,* 2004, 2008; Levin *et al.,* 2005 / AB_2618094	hybridoma supernatant
mAb MS77	plasmatocyte membrane, unknown epitope	mouse mAb	IgM	Willot *et al.*, 1994 / Beetz *et al.,* 2008	hybridoma supernatant
anti-Esterase	*Ephestia kuehniella* haemolymph esterase binds to cytoplasm of *M. sexta* spherule cells	rabbit immune serum		generous gift from G. Mann (Mann, 1992) / von Bredow *et al*., 2021	1:1,000
anti-*B. mori* Prophenoloxidase (PPO)	*Bombyx mori* prophenoloxidase, oenocytoids (*M. sexta*, *B. mori*)	rabbit immune serum		Iwama and Ashida, 1986 / Iwama and Ashida, 1986; Ashida *et al*., 1988; von Bredow *et al.*, 2021	1:1,000
anti-βGRP-1 3a	β-1,3-glucan recognition protein from *M. sexta* oenocytoids	rabbit immune serum		Ma and Kanost, 2000 / von Bredow *et al*., 2021	1:500

Lectin	Cell type	Carbohydrate specificity	Supplier / Cat. No.	References	Dilution
Arachis hypogaea lectin (PNA) TRITC, 2mg/ml	granules in granular cells granules and occasionally membrane of oenocytoids membrane of prohaemocytes	Gal, Galβ1-3GalNAc of N-glycans and glycolipids	Sigma-Aldrich, St. Louis, MI, USA / L3766	Nardi 2004; Kobayashi *et al*. 2014, Y. von Bredow *et al*., 2020; C. von Bredow *et al*., 2021	1:2,000

Secondary antibody	Conjugate	Host	Supplier	Cat. No. / RRID	Dilution
anti-mouse IgG/IgM	FITC	goat	Dianova GmbH, Hamburg, Germany	115-095-044 / AB_2338593	1:200
anti-rabbit IgG	DyLight 488	goat	Vector Laboratories, Burlingame, USA	DI-1488 / AB_2336402	1:2,000

monoclonal antibodies against haemocytes, it turned out that the morphologically distinct haemocyte classes also differently express certain epitopes which confirmed the earlier classifications in lepidopterans, e.g. *Manduca sexta* (Willot *et al*., 1994), *Pseudoplusia includens* (Gardiner and Strand, 1999), *Bombyx mori* (Nakahara *et al*., 2009).

In *M. sexta*, as in most lepidopteran insects, five classes of haemocytes were described based on morphological and ultrastructural criteria (Horohov and Dunn, 1982) which later was partly confirmed by antibodies labelling specific haemocyte types (Willot *et al*., 1994; Nardi *et al*., 2001; Beetz *et al*., 2004; Levin *et al*. 2005). Moreover, Horohov and Dunn (1982) provided information about approximate size of the haemocyte types and nuclear-to-cytoplasm ratio, but detailed values or ranges for each haemocyte type were missing.

Since specific markers are not always accessible it is often necessary to distinguish haemocyte types by light microscopy or easily accessible standard staining procedures. However, even trained researchers may encounter difficulties in haemocyte type determination due to similarities between cell types and variable size and shapes within the same cell type. Therefore, we present a set of morphological and cytometric criteria which can be used to identify the main haemocyte classes in *M. sexta* larvae by light microscopy and nuclear staining methods.

Methods

Insects

Manduca sexta of the colony maintained at the Institute of General Zoology and Developmental Biology, Justus-Liebig-Universität Gießen, Germany, were reared under standard conditions as outlined elsewhere (Beetz *et al*., 2004). Briefly, eggs were deposited on *Nicotiana rustica* plants, collected and incubated at 25 °C in polystyrene boxes containing a small block of artificial diet (modified after Yamamoto, 1969). Larvae were reared individually under long day light regime (16/8h light/dark).

Haemocyte collection and preparation of haemocyte monolayers

Two-day-old fifth-instar larvae (L5d2) were cleaned and anaesthetised by chilling on ice. An incision was made at a proleg or the abdominal horn and the haemolymph was collected in 5 mL ice cold anticoagulant saline (AC; 3.9 mM NaCl, 40 mM KCl, 146 mM sucrose, 0.1 % (w/v) polyvinylpyrrolidone 40, 8 mM EDTA, 9.5 mM citric acid, 27 mM trisodium citrate, 1.7 mM PIPES, pH 6.5). Haemolymph in AC was then centrifuged at 200 x g, 4 °C for 15 min. After discarding the supernatant, the haemocyte pellet was resuspended in 5 mL fresh AC and the procedure was repeated twice. The haemocyte pellet was then resuspended in approx. 1 mL insect cell culture medium (TC-100, Gibco ™ Thermo Fisher Scientific Inc., Waltham, MA, USA), and 15 µL cell suspension per well seeded on a 10-well glass slides (Thermo Scientific™ diagnostic slide, cat. number X1XER308B#MNZ, Thermo Fisher Scientific Inc., Waltham, MA, USA). After allowing the cells to settle for 45 min at room temperature in a humid chamber, the supernatant was removed and the cells were fixed by adding 3.5 % paraformaldehyde dissolved in calcium and magnesium free *M. sexta* saline (MS^-; 3.9 mM NaCl, 40 mM KCl, 146 mM sucrose, 0.1 % (w/v) polyvinylpyrrolidone 40, 1.7 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), pH, 6.5) for five min. After several rinses with Tris-buffered saline (TBS; 500 mM NaCl, 20 mM Tris, pH 7.5) the slides were stored at -20 °C.

Nucleus staining and antibody labelling

We used a toolset of haemocyte markers comprising monoclonal antibodies raised against larval haemocytes and immune sera against different proteins to identify the haemocyte types. Additionally, we combined labelling with these markers with the galactose-specific lectin PNA that has previously been shown to label different haemocyte types (Nardi, 2004; von Bredow et al., 2021). All markers, their specificity and known epitopes are listed in table 1.

Haemocytes were thawed and blocked with 5 % (v/v) normal goat serum (NGS), 3 % (w/v) bovine serum albumin (BSA), 0.05 % (w/v) sodium azide in TBS for one hour at room temperature. Incubation with primary antibodies (plasmatocyte specific antibodies: mAb MS13 (anti-plasmatocyte specific β-integrin, Willot *et al*., 1994, Beetz *et al*. 2004, 2008; Levin *et al*., 2005), or mAb MS77 (unknown epitope), (Willot *et al*., 1994; Beetz *et al*. 2008); granular cell specific antibody: mAb MS7 (unknown epitope), (Willot *et al*., 1994; Beetz *et al*., 2004, 2008); oenocytoid binding antibody: rabbit immune serum against *M. sexta* β-1,3-glucan recognition protein 1 (βGRP-1), diluted 1:500 (Ma and Kanost, 2000), generous gift from M. Kanost; spherule cells specific antibody: rabbit immune serum against *Ephestia kuehniella* haemolymph esterase, diluted 1:500 (Mann, 1992), generous gift from G. Mann, was performed overnight at 4 °C, followed by three rinses with TBS. Secondary antibody diluted in 5 % NGS, 3 % BSA, 0.05 % sodium azide in TBS, goat-anti-mouse IgG/IgM FITC-labelled, Dianova, Hamburg, Germany, diluted 1:200 or goat-anti-rabbit IgG DyLight 488-labelled, Vector Laboratories, Burlingame, CA, USA, diluted 1:2,000, respectively, together with lectin from *Arachis hypogaea* (peanut agglutinin, PNA, TRITC-labelled, Sigma-Aldrich, St. Louis, Mi, USA, diluted 1:2,000) was added for one hour at room temperature, followed by two rinses with TBS and DNA-labelling by incubation with 360 nM 4',6-diamidino-2-phenylindole (DAPI) in TBS for 15 min. After washing the cells with TBS for 10 min, the slides were mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA) and analysed with a BX60 fluorescence microscope with mounted camera (Altra 20 or XC10, both Olympus, Tokyo, Japan).

Determination of cell size, nucleus size and nucleus-to-cytoplasm ratio

Cell diameter, nucleus area and cytoplasm area of haemocyte types were selected manually from

photomicrographs and measured with the function "measure" of the open source software ImageJ 1.50e (Abràmoff *et al*., 2004, https://imagej.nih.gov/ij).

Haemocyte or nucleus length was determined as the highest dilatation of a single cell, cell width as the distance between cell boundaries orthogonally to the length-axis. Length-to-width ratio of haemocytes or nuclei was calculated by dividing length by width.

The nucleus-to-cytoplasm ratio was calculated as nucleus area divided by cytoplasm area (cell area minus nucleus area). This method was only used for non-spread haemocytes due to the high degree of polymorphism of spreading haemocytes.

The relative DNA-content was calculated by measuring the integrated density and area of DAPI-stained nuclei with ImageJ 1.50e (Abràmoff *et al*., 2004). For each photomicrograph, five non-dividing, typical granular cells, distinguished by morphology and PNA-positive granular inclusions were measured to receive a baseline intensity of diploid cells (Nardi *et al*., 2003). Background fluorescence was subtracted and fluorescence intensity was calculated according to the method described by McCloy *et al*. (2014). Relative DAPI-fluorescence intensity was calculated by dividing DAPI-intensity of sampled cells by mean DAPI-intensity of diploid granular cells.

Each analyses was performed on at least three independent biological replicates.

Statistical analysis

Tests for significance were performed with R version 3.6.2 (R Core Team, 2014). The cytometric values cell diameter (length and width), nucleus diameter, cell area, nucleus area, cell length-to-width-ratio, and nucleus length-to-width-ratio were tested for differences between the cell types using one-way-ANOVA with post-hoc pairwise comparison (Tukey-test). Differences were classified as significant at $p \leq 0.05$.

The relative DAPI-intensities and the nucleus-to-cytoplasm-ratios were compared between the haemocyte types using Mann-Whitney-*U*-test, with $p \leq 0.05$ considered as significant difference.

Results

Morphology of haemocyte types and specific labelling by antibodies and the lectin PNA

Binding characteristics of the antibodies and the lectin PNA on haemocytes are shown in Fig. 1, and summarised in table 1. Granular cells were specifically labelled by the mAb MS7 (Fig. 1 A'), plasmatocytes were specifically bound by the mAb MS13 (Fig. 1 B), and the mAb MS77 (Fig. 1 E'), respectively. Oenocytoids were the only haemocyte type labelled by anti-βGRP-1 immune serum (Fig. 1 C', table 1). The immune serum against *E. kuehniella* haemolymph esterase (anti-esterase) is the only known available antibody binding exclusively to spherule cells but no other haemocyte type (Fig. 1 D'). Granular inclusions of granular cells (Fig. 1 A'') and few granules in oenocytoids were PNA-positive (Fig. 1C'', 1Ei'). Furthermore, some haemocytes were labelled with PNA at the cell surface. These cells were spherule cells, which had a slightly labelled cell surface (Fig. 1 D''). More extensively stained were non-spread plasmatocytes (Fig. 1 E''), oenocytoids and putative prohaemocytes (Fig. 1 E_{ii}', PNAm+).

Small spread haemocytes which were similar in shape to spread plasmatocytes did not share most key characteristics of plasmatocytes. The nuclei of small spread haemocytes were small and had a round shape, whereas plasmatocytes had large and mostly irregularly formed nuclei (compare the nuclei of sprGR to the nuclei of rPL or sprPL in Fig. 1). Antibody labelling revealed no antigenic similarities between the small spread cells and plasmatocytes. Plasmatocytes were labelled by MS13 and MS77, whereas small spread haemocytes were not (compare labelling pattern of sprGR to labelling pattern of rPL or sprPL in Fig. 1 B', and sprGR with rPL in Fig. 1 E'). Furthermore, the granular cells labelling mAb MS7 bound to small spreading cells (Fig. 1A'), and PNA labelled granular inclusions, too, as opposed to sprPLs (Fig. 1 A'', B''). Oenocytoid markers (anti-βGRP-1, Fig. 1 C'; anti-prophenoloxidase, data not shown) and the spherule cell marker anti-esterase (Fig. 1 D') did not label the small spread haemocytes. Additionally, the relative DAPI intensity fitted to granular cells rather than plasmatocytes or other cell types (Fig. 3 A), indicating that these small spread cells resemble a pseudopodia forming, spread granular cell population, which we termed spreading granular cells (sprGR).

Confusingly, oenocytoids and round plasmatocytes sometimes share the same size and shape (compare appearance of OEs with appearance of rPLs in Fig. 1), and might therefore be misidentified. Two characteristics were identified to reliably identify each haemocyte type: first, the oenocytoid cytoplasm does sometimes appear in light microscopy more granulated (a weak characteristic), and second, the size and shape of the nuclei (Fig. 1, Fig. 2 D, E), but not of the DNA-content (Fig. 3 A), differed largely between oenocytoids and round plasmatocytes. Oenocytoid nuclei were round while plasmatocyte nuclei were most often irregularly-oval formed (compare the nucleus of an OE to the nucleus of rPLs in Fig. 1 C''' and Fig. 1 B'''), and normally a larger portion of the cytoplasm is visible in oenocytoids than in round plasmatocytes, the latter possess a larger nucleus and only sparse cytoplasm (Fig. 3 B).

Size and shape of the haemocyte types

For the morphological description of the haemocyte types, the cell area, length (maximum distance) and width (minimum distance) and the length-to-width ratio were determined. All data are shown in Fig. 2 (cell and nuclei diameter and length to width ratios), Fig. 3 (relative DAPI-intensity of the nuclei and nucleus-to-cytoplasm-ratios), Fig. 4 (typical appearance of haemocyte types and nuclei) and supplementary Table S1.

Granular cells occur in two different morphs, either and most abundant as unspread, round cells (abbreviated as GR as it is the common form), or, as evidenced in this work, as spreading cells (sprGR). The round granular cells (GR) ranged in diameter from 5.0 µm to 10.1 µm, with a mean

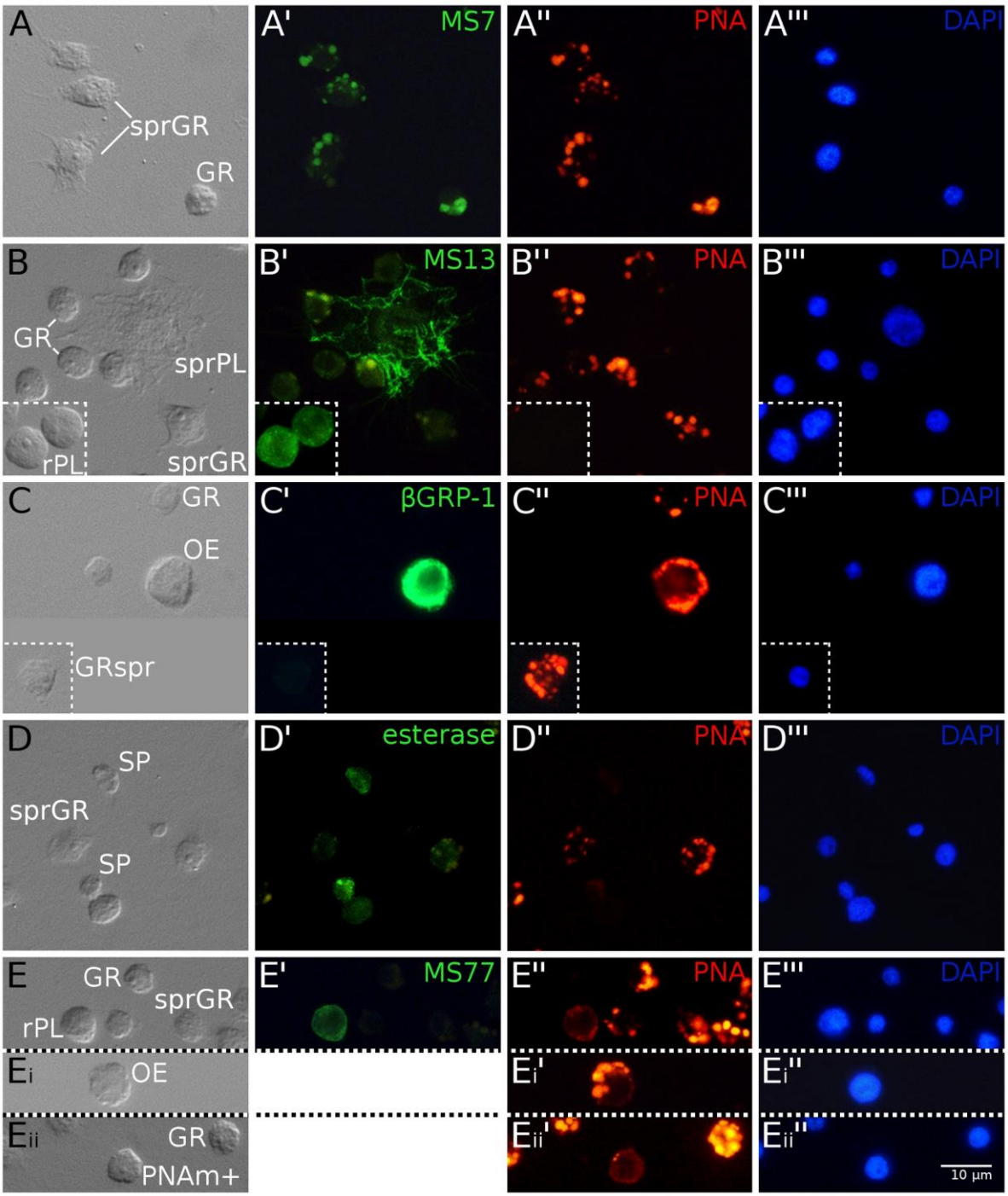

Fig. 1 Antibody- and PNA-labelling of haemocyte types and corresponding nuclei morphology. A-A''': Granular cells and spread granular cells have MS7- and PNA-positive cytoplasmic inclusions (granules). Nuclei of round granular cells are round, nuclei of spread granular cells are round to oval. B-B''': Plasmatocytes can be round or spread, both forms labelled by MS13. Plasmatocytes contain no PNA-positive granules. Nuclei of both spread and round plasmatocytes are eccentric or oval in shape. C-C''': Oenocytoids are positive for β-GRP-1 and may contain PNA-positive granules. Nuclei are round and larger than nuclei of granular cells. D-D''': Anti-*E. kuehniella* haemolymph esterase immune serum labels spherule cells, PNA slightly labels their cell surface. Spherule cells are round (lower SP) to oval or rod-shaped (upper SP), their nuclei are mostly elongated or irregularly formed (D'''). E-E''': A round plasmatocyte (labelled by MS77) exhibits labelling of the cell surface by PNA. Ei-Ei'': Oenocytoid with PNA-positive granules and PNA cell surface staining. Eii-Eii'': A round, small haemocyte labelled with PNA at the cell surface. Note the lesser granulation of the cytoplasm when compared to the granular cell and the absence of PNA-positive granules. The nucleus is round in shape and slightly larger than that of the granular cell. Abbreviations: GR, granular cell; OE, oenocytoid; PL, plasmatocyte; PNAm+, haemocyte with PNA-positive cell membrane; rPL, non-spread plasmatocyte; SP, spherule cell; sprGR, spread granular cell. The scale bar represents 10 µm

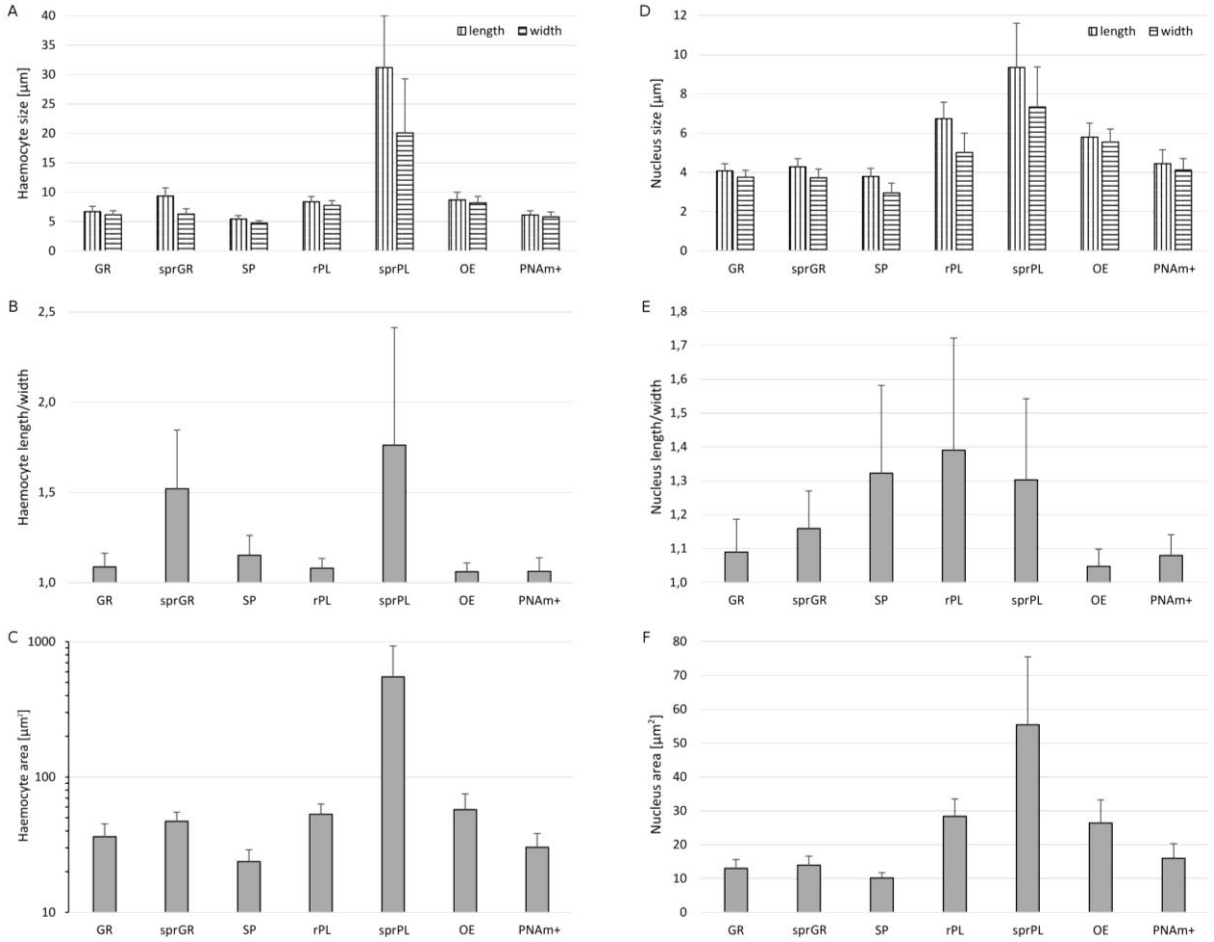

Fig. 2 Size, length-to-width-ratio and area of different haemocyte types and their nuclei. A: Haemocyte length and width of each haemocyte type. B: Haemocyte length-to-width ratio depicting the differences in shape of the haemocyte types. C: Area occupied by each haemocyte type. The y-axis (area) is logarithmized. D: Nucleus length and width of each haemocyte type. E: Nucleus length-to-width ratio depicting the differences in nucleus shape of the haemocyte types. F: Area occupied by the nuclei of each haemocyte type. Bars depict mean values, whiskers depict the standard deviation. Statistical analysis (ANOVA with post-hoc Tukey-test) is summarised in Supplementary table S2 and S3. Abbreviations: GR, granular cell; OE, oenocytoid; sprGR, spread granular cell; sprPL, spread plasmatocyte; PNAm+, haemocyte with PNA-positive cell membrane; rPL, non-spread plasmatocyte; SP, spherule cell

diameter of 6.1 µm, SD 0.9 (width) and 6.7 µm, SD 0.9 (length). The area covered by these cells ranged from 20.7 µm^2 to 59.9 µm^2, with a mean of 36.3 µm^2, SD 8.8. With a range of length-to-width-ratio (L:W) between 1.0 and 1.3, and a mean L:W of 1.1, these cells are best described as round in shape. The spread granular cells (sprGR) differed from the round form by projecting numerous pseudopodia, and in consequence by their altered cytometric values. They were larger in their length (i.e. maximum diameter) and occupied a larger area (between 32.6 µm^2 and 64.8 µm^2, mean 46.1 µm^2, SD 7.9) than round granular cells. Their diameter measured between 4.3 µm and 12.8 µm, with mean diameter of 6.3 µm, SD 0.9 (width) and 9.3 µm, SD 1.4 (length). The L:W was between 1.0 (evenly spread cells without polar extensions) and 2.9 (highly polar spread cells projecting cytoplasmic protrusions preferably along a single axis), with a mean L:W of 1.5, SD 0.3. Numerous granular inclusions were frequently visible in the cytoplasm of both round and spread granular cells by differential interference contrast microscopy, many of them were labelled by the lectin PNA and the mAb MS7 (Fig. 1 A', A'').

Spherule cells (SPs) were the smallest cell type with diameter between 3.8 µm and 6.5 µm, with a mean diameter of 4.7 µm, SD 0.4 (width) and 5.4 µm, SD 0.6 (length). The cell area occupied by these cells was between 15.5 µm^2 and 39.5 µm^2, with a mean area of 23.7 µm^2, SD 5.3. Their shape ranged from round (L:W of approx. 1.0) to elongated (L:W of ≥ 1.3). The mean L:W of 1.16 indicates the morphological variability. Large spherules, eponymous for this haemocyte type, were found in the majority of the spherule cells, but were not

always clearly visible by means of light microscopy (cf. the upper SP with large spherules and the lower SP lacking obvious spherules in Fig. 1 D).

Plasmatocytes occur in two main forms, round (non-spread) or spread. Intermediate forms can also occur, which are characterised by a large round cell body containing the nucleus and pseudopodia projecting from there, indicating a transition from the round to the spread form (not shown). The round plasmatocytes (rPL) were relatively large round haemocytes with diameter ranging from 6.5 µm to 10.4 µm, with a mean diameter of 7.75 µm, SD 0.8 (width) and 8.36 µm, SD 0.9 (length). The cell area covered between 35.3 μm^2 and 75.3 μm^2, mean area 53.1 μm^2, SD 10.1. The L:W ranged between 1.0 and 1.2, mean L:W 1.1, SD 0.1, which mirrors their round shape. The spread plasmatocytes (sprPL) differed in their dimensions from the unspread form, with a diameter between 7.5 µm and 51.1 µm, mean diameter 20.1 µm, SD 9.2 (width) and 31.2 µm, SD 8.8 (lenght). The cell area was between 133.5 μm^2 and 1,787.1 μm^2, mean area 550.5 μm^2, SD 378.6. Spreading as a result of pseudopod projection led to L:W ranging between 1.1 (isotropic spread plasmatocytes, which appear nearly round, “fried egg shape”) and 3.7 (highly polarised, anisotropic spread cells, with pseudopods projecting mainly to one or two directions along a single axis).

Oenocytoids (OEs) were relatively large, round cells, with a diameter measuring between 6.2 µm and 12.8 µm. The mean diameter was 8.2 µm, SD 1.1, (width) and 8.7 µm, SD 1.3 (length). Oenocytoids covered areas between 34.2 μm^2 and 123.3 μm^2, with a mean of 57.6 μm^2, SD 17.6. Owing to their round to oval appearance, the L:W ranged between 1.0 and 1.2, with a mean L:W of 1.1, SD 0.1.

Putative prohaemocytes (ProHC), small round haemocytes which were identified by labelling of the cell membrane with PNA (PNAm+) and a relatively large area occupied by the nucleus, measured between 4.6 µm and 8.4 µm, with a mean diameter of 5.8 µm, SD 0.8 (width) and 6.1 µm, SD 0.7 (length). They covered between 19.7 μm^2 and 54.9 μm^2, with a mean area of 30.3 μm^2, SD 8.0. The L:W was between 1.0 and 1.3, with a mean L:W of 1.1, SD 0.1, mirroring their overall round shape.

Length and width of each of the round haemocytes types did not differ largely within individual cells, resulting in a length-to-width ratio close to 1 (Fig. 2 A, B). This characteristic was found in granular cells, round plasmatocytes, oenocytoids and putative prohaemocytes. Spherule cells exhibited a slightly elevated length-to-width-ratio. Spread granular cells as well as spread plasmatocytes showed a remarkable difference between their maximum expansion and minimum expansion, resulting in a mean length-to-width-ratio above 1.5 (Fig. 2 B).

Nuclear morphology, relative DNA content and nucleus-to-cytoplasm-ratio of haemocyte types

Haemocytes exhibited haemocyte type specific differences of size, shape and DNA content of their respective nuclei. As a rule of thumb, round haemocytes had a similarly round nucleus, while spread or otherwise deformed haemocytes had oval or irregularly formed nuclei. An exception were the round plasmatocytes with mostly irregularly shaped nuclei. All data can be found in fig 2 D - F, Fig. 3, and supplementary Table S1.

Nuclei of the round granular cells measured between 3.1 µm and 5.3 µm in diameter, with a mean of 3.8 µm, SD 0.4 (width) and 4.1 µm, SD 0.4 (length). The nucleus area occupied between 8.4 μm^2 and 19.1 μm^2, mean area 13.0 μm^2, SD 2.6. The nuclear L:W ranged between 1.0 and 1.4, mean 1.1, SD 0.1, which defines the nuclei as mostly round. The relative DAPI-intensity was between 0.7-fold and 2.3-fold of diploid granular cells, with a median of 1.1, interquartile range (IQR) 0.5. Nuclei of the spread granular cells had a similar range of relative DAPI-intensity as round granular cells, ranging from 0.6-fold to 2.2-fold of diploid granular cells, median 1.3, IQR 0.7. The nucleus-to-cytoplasm-ratio (N:C) was not determined for this cell type.

Nuclei of the spherule cells had a remarkably low DAPI-intensity at the minimum level measured with 0.34-fold intensity, the highest intensity, however, was 2.0-fold compared to granular cells. The median DAPI-intensity also was lower than expected at 0.76, IQR 0.3. The N:C ranged between 0.46 and 2.51, with a median N:C of 1.0, IQR 0.6.

Nuclei of the round plasmatocytes exhibited always an elevated relative DAPI-intensity, from 1.4-fold to 6.3-fold, median 3.1, IQR 0.6. This implies a relatively high DNA-content compared to diploid granular cells. The N:C measured between 0.45 and 2.73, median 1.2, IQR 0.5. Spread plasmatocytes had an even higher relative DAPI-intensity between 1.8-fold and 7.1-fold, median 3.8, IQR 0.7. The N:C was not determined for this cell type.

The nuclei of the oenocytoids had a relative DAPI-intensity between 1.1 and 7.1, median 3.0, IQR 1.1. Their N:C ranged between 0.4 and 1.4, median 0.9, IQR 0.4, which indicates that the area occupied by the nucleus was mostly smaller than the area of the cytoplasm.

Putative prohaemocytes (PNAm+ cells) exhibited nuclei with a relative DAPI-intensity between 0.7 and 2.6, median 1.3, IQR 0.5. Their N:C was between 1.0 and 2.8, with a median N:C of 1.6, IQR 1.0. Therefore, these cells had a nuclear area exceeding the area covered by the cytoplasm.

Dichotomous identification key for M. sexta *haemocyte types*

From the data presented in this paper, we derived a key to each haemocyte type in *M. sexta* larvae, which can be applied to fixed adhering haemocytes labelled with standard DNA-stains like DAPI and light microscopy, preferentially using differential interference contrast. Please note that some characteristics such as granulation of the cytoplasm may be obscured depending on the microscopy technique used. An overview of typical haemocyte type shape and nuclei is given in Fig. 4. A graphical short-key is shown in Fig. 5. We recommend to refer to the detailed description presented in the main text below, and summarised in Supplementary table S1 to enhance the accuracy of determination.

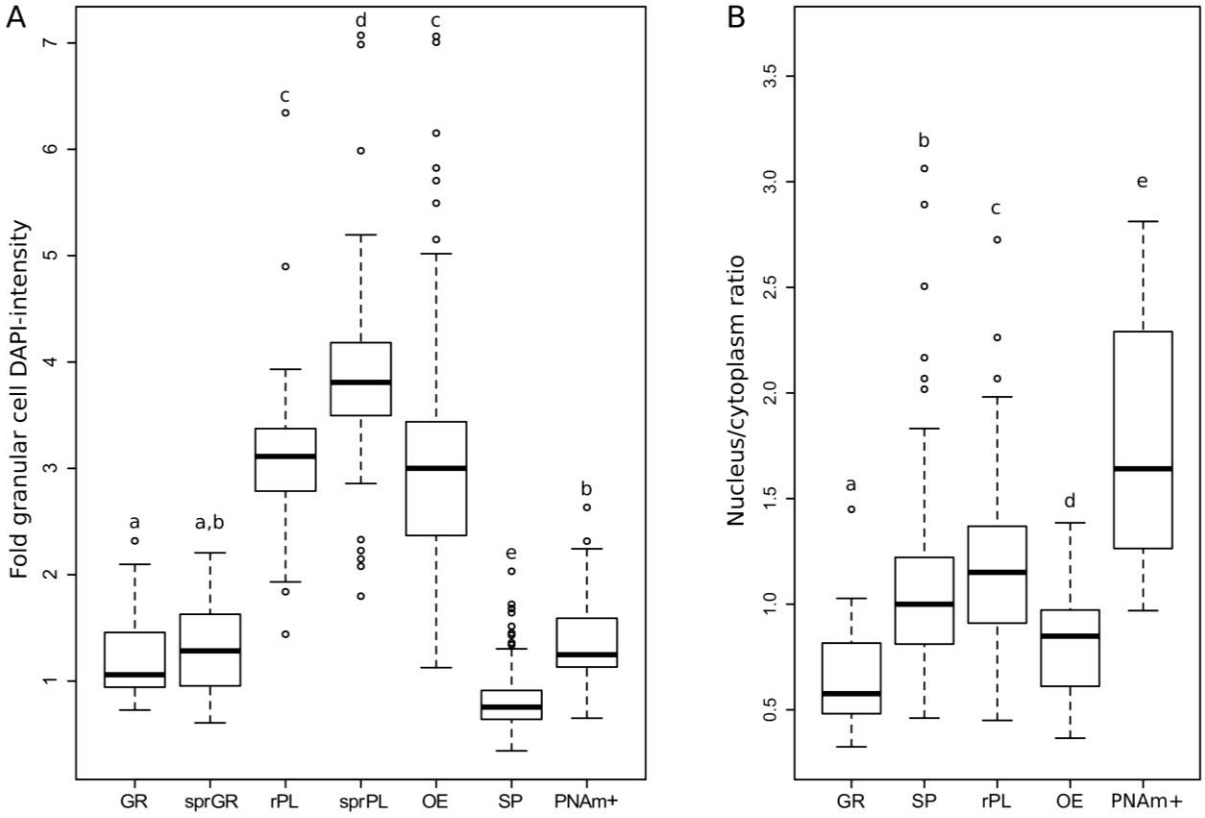

Fig. 3 Relative DAPI-intensity and nucleus-to-cytoplasm-ratio of different haemocyte types. A: The DAPI-intensity of the different haemocyte types in relation to the intensity of typical diploid granular cells. Granular cells (GR), spread granular cells (sprGR), spherule cells (SP) and PNA cell membrane positive putative prohaemocytes (PNAm+) do seldom exceed a two-fold granular cell DAPI-intensity, whereas plasmatocytes, both spread (sprPL) and round (rPL) as well as oenocytoids (OE) regularly contain nuclei with a more than two-fold higher DAPI intensity, indicating polyploidy of these cell types. B: The nucleus-to-cytoplasm area ratio of non-spread haemocyte types. Horizontal bars within each box depict the median, upper and lower quartile are depicted by the upper and lower borders. Whiskers represent the 1.5-fold interquartile range, outliers are depicted by empty circles. Different lower-case letters depict significant differences at $p \leq 0.05$, Mann-Whitney-*U*-test. These data were also used in another context in von Bredow *et al*. (2021) to clarify the identity of haematopoietic organ cells. Abbreviations: GR, granular cell; OE, oenocytoid; sprGR, spread granular cell; sprPL, spread plasmatocyte; PNAm+, haemocyte with PNA-positive cell membrane; rPL, non-spread plasmatocyte; SP, spherule cell

Cell spread, with pseudopods (1); cell not spread (2).
1) Maximum cell diameter > 13 µm (3), smaller (4)
2) Cell not spread. Cell shape oval, nucleus round with smooth edge (5), other (6)
3) Spread plasmatocyte (sprPL). Large spread cell (diam. 7.5 – 51 µm), nucleus large (diam. 4.7 – 11 µm) and mostly oval or irregular (nuc. L:W 1.0 – 1.9), polyploid (rel. DAPI-intens. 1.8 – 7.1-fold of diploid granular cells).
4) Spread granular cell (sprGR). Small spread cell (diam. 4.3 – 12.8 µm), nucleus small (diam. 2.8 – 5.1 µm) and round to oval (nuc. L:W 1.0 – 1.5), diploid or tetraploid (rel. DAPI-intens. 0.6 – 2.2-fold of diploid granular cells).
5) Oenocytoid (OE). Medium to large sized cell (diam. 6.2 – 12.8 µm), with round to oval shape (cell L:W 1.0 – 1.2) and smooth edge, nucleus round to slightly oval (nuc. L:W 1.0 –1.2), mostly polyploid (rel. DAPI-intens. 1.1 – 7.1), cytoplasm area mostly larger than nuclear area (N:C 0.4-1.4).
6) Cell shape irregularly, elongated, comma-shaped or vermiform, often with large cytoplasmic inclusions (7), other (8)
7) Spherule cell (SP). Small, irregularly formed cell (cell diam. 3.8 – 6.5 µm, cell L:W 1.0 – 1.4), often with visible spherules. Nucleus small, irregularly shaped (sickle, triangle, elongated, irregularly oval; diam. 1.6 – 4.9 µm, nuc. L:W 1.0 – 3.9) with mostly larger cytoplasm than nucleus (N:C 0.5 – 2.5). Diploid to tetraploid (rel. DAPI-intens. 0.3 – 2.0).
8) Nucleus irregularly shaped or oval (9), nucleus round (11)
9) Max. nucleus diameter 3 – 5 µm, max. cell diameter 4 – 6.5 µm (7), max. nucleus diameter 5 – 10 µm, max. cell diameter > 6.5 µm (10)
10) Round plasmatocyte (rPL). Large, mostly irregular-round (cell diam. 6.5 – 10.4 µm, cell L:W

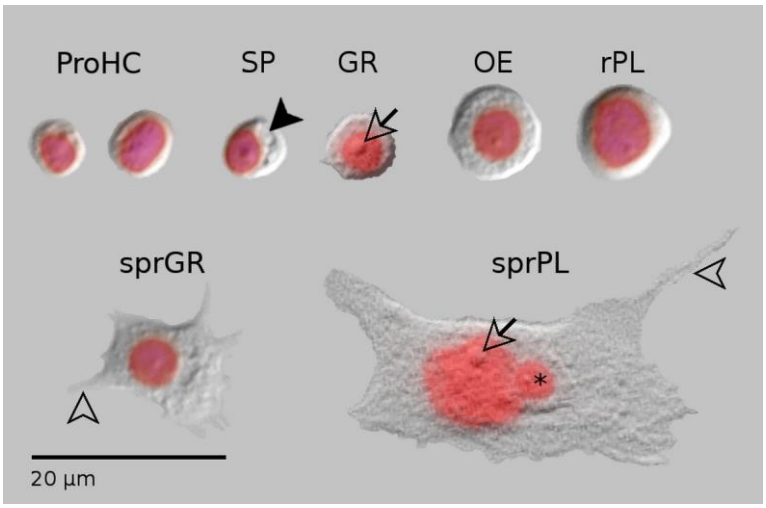

Fig. 4 Typical appearance and nucleus shape of the larval haemocyte types in *M. sexta*. Differential interference contrast microscopy merged with fluorescence microscopy of the nuclei (red). Numbers in square brackets correspond to the numbers in the dichotomous identification key (text based, and Fig. 5). Upper row from left to right: a typical prohaemocyte (ProHC, [13]) with small nucleus and sparse cytoplasm and a putative prohaemocyte with a large nucleus; a spherule cell (SP, [7]) with slightly deformed nucleus and typical large cytoplasmic inclusion (spherule, solid arrowhead); a granular cell (GR, [12]) with granulated cytoplasm, the empty arrow marks the nucleolus; an oenocytoid (OE, [5]) with typical round nucleus; a round plasmatocyte ([10] rPL) with ovoid nucleus. Lower row from left to right: A spread granular cell (sprGR [4]), the empty arrowhead marks a typical cytoplasmic protrusion; a spread plasmatocyte (sprPL [3]) with a large, irregularly shaped nucleus, the empty arrowhead marks a thin cytoplasmic protrusion. The scale bar equals 20 µm

1.0 – 1.2) cell with smooth edge. Nucleus large (nuc. diam. 3.0 – 9.2 µm), excentric to elongated (nuc. L:W 1.0 – 2.9), with uneven edge. Polyploid (rel. DAPI-intens. 1.4 – 6.3), nuclear area mostly exceeding cytoplasmic area (N:C 0.5 – 2.7).

11) a) Cytoplasm granulated, N:C < 1.0, nucleus diameter 3-5 µm, cell diameter typically 6 – 8 µm, 2n or 4n (12); or b) N:C < 1.0, nucleus diameter 4 – 8 µm, cell diameter 6 – 13 µm, typically > 2n (5); or c) N:C > 1.0, nucleus diameter 3 – 6 µm, cell diameter 4-9 µm, 2n or 4n (13)

12) Granular cell (GR). Small to medium sized (cell diam. 5.0 – 10.1 µm) round to oval (cell L:W 1.0 – 1.3) cell with granulated cytoplasm, often with uneven edge. Nucleus small (nuc. diam. 3.1 – 5.3 µm) and round (nuc. L:W 1.0 - 1.4), diploid to tetraploid (rel. DAPI-intens. 0.7 – 2.3). Cytoplasm area most often exceeds nuclear area (N:C 0.3 – 1.5).

13) Prohaemocyte (ProHC). Small to medium sized round cell (cell diam. 4.6 – 8.4 µm, cell L:W 1.0 – 1.3) with smooth edge. Nuclear diameter between 3.1 and 6.0 µm, more or less round (nuc. L:W 1.0 – 1.2), diploid to tetraploid (rel. DAPI-intens. 0.7 – 2.6), with a nuclear area exceeding the cytoplasm area (N:C 1.0 – 2.8).

Discussion

Albeit there exist numerous definitions of haemocyte types in insects, and it is consensus that five main haemocyte types exist in Lepidoptera (Arnold, 1982), a clear definition of the haemocyte types in *M. sexta* based on cytometric data has not been published yet. With a defined tool set of monospecific markers for each haemocyte type, foremost monoclonal antibodies raised against whole larval haemocytes (Willott *et al*., 1994), immune sera against specific proteins (Iwama and Ashida, 1986; Mann, 1992; Ma and Kanost, 2000), and the lectin PNA, we were able to clearly distinguish each haemocyte type and determined their key characteristics. We found that many haemocyte types exhibit overlapping characteristics, but when these are combined, a definitive separation of each type was possible.

The most obvious difference occurred between spreading cells, which expand numerous pseudopodia, and unspread cells lacking pseudopodia. Initially it was unclear whether the small spreading cells, which may occur on short-time cultured haemocyte monolayers, represent small plasmatocytes or another haemocyte type. Due to the unambiguous labelling pattern with granular cell markers labelling the small but not the large spread haemocytes, and the unambiguous labelling pattern with plasmatocyte labelling antibodies labelling only the large spread haemocytes, it turned out that the small spreading haemocytes represent a spread form of granular cells. This finding was later confirmed by both the size and shape of the nuclei, which fits to granular cells quite good, and moreover by the DNA-content, which did not exceed that of the typical round granular cells. A clear cut between spread granular cells and spread plasmatocytes could be made by the maximum cell diameter: Spread plasmatocytes

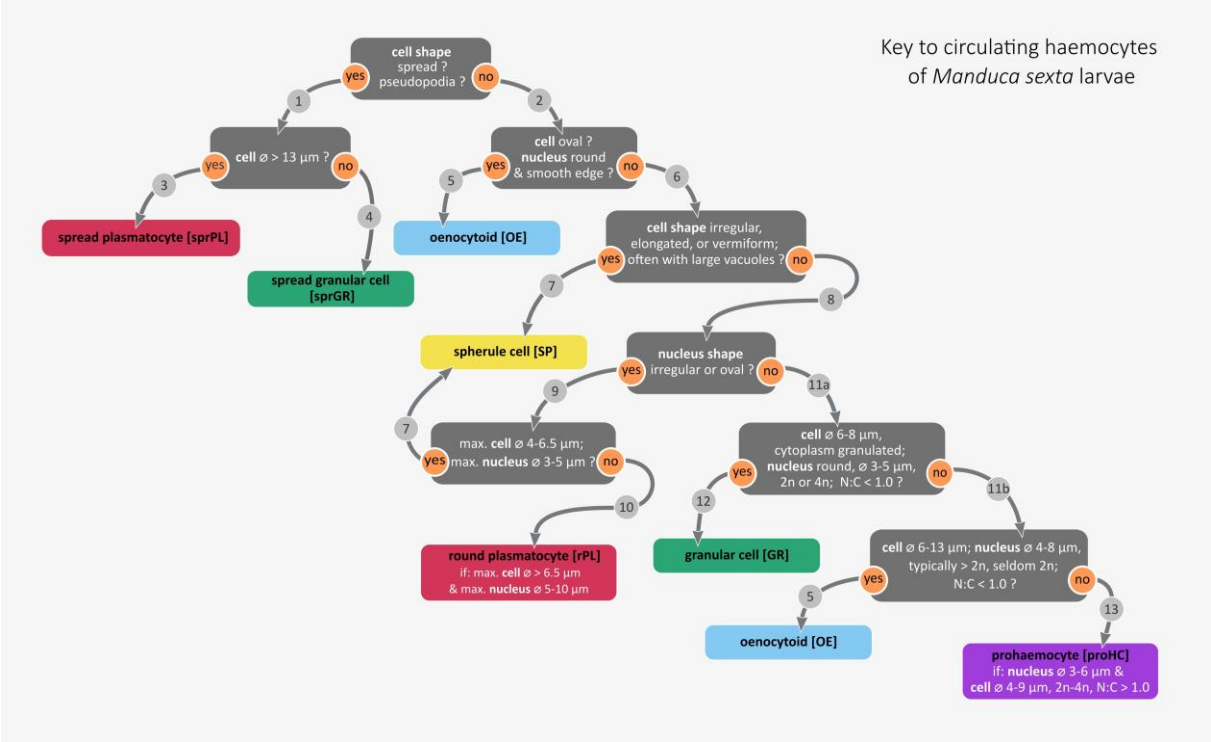

Fig. 5 Simplified dichotomous key to circulating larval *M. sexta* haemocytes. The main determination criteria are given as yes/no questions (grey boxes). The cell types appear in colour-coded boxes, where same colour indicates same cell type. Cell types occuring in different morphs (spread and non-spread) are indicated by same colour. Numbers correspond to the text-based dichotomous key presented in the main text. Please verify determination by referring to Fig. 4 and the detailed characteristics listed in the main text and Supplementary table S1

always exhibited a maximum diameter of ca. 18 µm or more, while spread granular cells were never longer than 13 µm. This characteristic, however, could vary, e.g. when the cells are cultured over a shorter or longer period of time before fixation or when other substrata than glass slides are present (e.g. poly-L-Lysin-coated slides). Therefore, these data should be carefully validated separately by each researcher before it can be applied.

The ability of adhering granular cells to spread on glass surfaces and forming lamellipodia is known from several lepidopteran insect species. For example *Bombyx mori* (Yamashita and Iwabuchi, 2001), *Pseudoplusia includens (*Lavine and Strand, 2002), *Mythmina unipunctata* (Ribeiro and Brehélin, 2006), *Galleria mellonella* (İzzetoğlu, 2012), but we found no literature describing granular cell spreading in *M. sexta*. Owing to the overall seldom appearance of granular cells showing spreading behaviour, which does neither occur in a high number of granular cells nor in each preparation of haemocytes (data not shown), we conclude that these cells may have been overlooked or ignored, or misinterpreted as small plasmatocytes in previous works. Which mechanisms underlies the spreading behaviour of granular cells is largely unknown. The plasmatocyte spreading peptide does not influence spreading of granular cells (Eleftherianos *et al*., 2009). It is, however, imaginable that spreading of granular cells is a consequence of degranulation, which leads to a decrease of the cell volume of the granular cells and could promote cell shape changes. Additionally, spreading could be influenced by the immune status of the animal.

Within the non spreading haemocyte types, i.e. round granular cells, round plasmatocytes, spherule cells and oenocytoids, each cell type exhibited a unique combination of characteristics allowing a determination without the use of specific markers. With simple DNA-staining and light microscopy, each cell type can be determined based on its combination of cytometric characteristics. While traditional characteristics like “granulated cytoplasm” or ”spherules in the cytoplasm” may be misleading, characteristics like size and shape of cell and nucleus, combined observation of DNA-content and nucleus-to-cytoplasm-ratio allow a relatively reliable determination of each cell. By comparison of each cell to be analysed with the dichotomous key provided in this paper it is possible to determine each haemocyte type.

It must be taken care whether the guide is suitable to compare haemocyte types of stages other than L5, if the culturing conditions influence the shape of the haemocytes to be compared, or if

physiological conditions such as immune stimulation or starvation may influence the haemocyte appearance. Therefore, we recommend for each haemocyte type determination under other conditions than stated in this paper to assess the morphometric data to ensure comparable results.

Highlights

Cytometric analyses of *M. sexta* larval haemocyte types allow their identification

A dichotomous identification key for *M. sexta* haemocytes was created

Ploidy levels of haemocyte types were identified by relative DAPI-intensity

Nucleus-to-cytoplasm ratio was measured for each unspread haemocyte type

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Competing interests

The authors declare that they have no competing interests.

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Authors contributions

CvB conceived the study, performed experiments and data analyses, and wrote the manuscript; TET provided lab utilities, antibodies and edited the manuscript.

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Supplementary table S1 Cytometric data for each *M. sexta* haemocyte type

	GR			sprGR			SP			rPL			sprPL			OE			PNAm+		
	min	**max**	**mean±SD**	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**	**min**	**max**	**mean±SD**
Cell l [µm]	5.54	10.11	6.67±0.92	6.86	12.84	9.34±1.38	4.12	6.49	5.43±0.60	6.73	10.37	8.36±0.91	17.88	50.96	31.19±8.79	6.47	12.77	8.66±1.33	4.99	8.41	6.13±0.72
Cell w [µm]	5.00	8.33	6.14±0.71	4.25	8.37	6.27±0.91	3.76	5.58	4.72±0.43	6.52	9.30	7.75±0.80	7.48	42.13	20.08±9.21	6.21	10.80	8.16±1.13	4.61	8.36	5.80±0.83
Cell L:W	1.01	1.29	1.09±0.08	1.04	2.88	1.52±0.33	1.01	1.37	1.15±0.11	1.00	1.19	1.08±0.05	1.05	3.68	1.76±0.65	1.00	1.19	1.06±0.05	1.00	1.26	1.06±0.08
Cell a [µm^2]	20.65	59.98	36.30±8.81	32.60	64.79	46.06±7.91	15.54	39.49	23.72±5.32	35.34	75.27	53.05±10.14	133.52	1,787.14	550.49±378.63	34.21	123.26	57.56±17.57	19.66	54.93	30.30±8.00
Nuc. l [µm]	3.53	5.27	4.07±0.37	3.43	5.09	4.29±0.41	3.09	4.85	3.79±0.41	5.30	9.23	6.73±0.84	7.45	14.32	9.74±1.51	4.67	7.70	5.80±0.71	3.25	6.03	4.44±0.71
Nuc. w [µm]	3.14	4.39	3.75±0.36	2.78	4.51	3.72±0.44	1.58	3.88	2.95±0.50	3.00	7.42	5.01±0.99	4.69	11.17	7.69±1.66	4.28	7.13	5.54±0.66	3.07	5.38	4.11±0.59
Nuc. L:W	1.00	1.43	1.09±0.10	1.02	1.47	1.16±0.11	1.00	2.09	1.33±0.26	1.02	2.85	1.39±0.33	1.02	1.92	1.30±0.24	1.00	1.24	1.05±0.05	1.00	1.21	1.07±0.06
Nuc. a [µm^2]	8.44	19.09	13.00±2.60	9.33	20.28	13.94±2.66	7.73	14.69	10.16±1.55	19.97	40.54	28.37±5.12	21.84	103.79	55.40±20.06	16.42	44.97	26.38±6.80	10.73	29.09	15.95±4.33
	GR			**sprGR**			**SP**			**rPL**			**sprPL**			**OE**			**PNAm+**		
	min	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**	**min**	**max**	**Mdn±IQR**
Rel. DAPI-int.	0.73	2.32	1.06±0.51	0.61	2.21	1.28±0.67	0.34	2.03	0.76±0.27	1.44	6.34	3.11±0.59	1.80	7.07	3.81±0.69	1.13	7.06	3.00±1.07	0.65	2.63	1.25±0.46
N:C	0.32	1.45	0.58±0.34	N.D.	N.D.	N.D.	0.46	2.51	1.02±0.61	0.45	2.73	1.17±0.47	N.D.	N.D.	N.D.	0.37	1.39	0.85±0.37	0.97	2.81	1.64±1.04

Abbreviations: a, area; l, length; L:W, length-to-width ratio; Nuc., nucleus; w, width; mean±SD, mean ± standard deviation; Mdn±IQR, median ± interquartile range; N:C, nucleus-to-cytoplasm-ratio. Numbers of cells and animals analysed for cell length, width and cell L:W: GR cells n = 36, animals n = 3, sprGR cells n = 47, animals n = 3, rPL cells n = 33, animals n = 3, sprPL cells n = 33, animals n = 3, OE cells n = 33, animals n = 3, SP cells n = 27, animals n = 3, PNAm+ cells n = 21, animals n = 4. Numbers of cells and animals analysed for cell area: GR cells n = 48, animals n = 4, sprGR cells n = 53, animals n = 4, rPL cells n = 35, animals n = 4, sprPL cells n = 32, animals n = 4, OE cells n = 34, animals n = 4, SP cells n = 36, animals n = 4, PNAm+ cells n = 17, animals n = 4. Numbers of cells and animals analysed for nucleus length, width and nucleus L:W: GR cells n = 35, animals n = 3, sprGR cells n = 52, animals n = 3, rPL cells n = 41, animals n = 3, sprPL cells n = 31, animals n = 3, OE cells n = 35, animals n = 3, Sp cells n = 34, animals n = 3, PNAm+ cells n = 17, animals n = 4. Numbers of cells and animals analysed for nucleus area: GR cells n = 44, animals n = 4, sprGR cells n = 50, animals n = 4, rPL cells n = 33, animals n = 4, sprPL cells n = 38, animals n = 4, OE cells n = 36, animals n = 4, SP cells n = 38, animals n = 4, PNAm+ cells n = 18, animals n = 4. Numbers of cells and animals analysed for nucleus-to-cytoplasm-ratio (N:C): GR cells n = 62, animals n = 5, rPL cells n = 71, animals n = 3, OE cells n = 55, animals n = 7, SP cells n = 120, animals n = 5, PNAm+ cells n = 33, animals n = 5. Numbers of cells and animals analysed for DAPI-intensity: GR cells n = 86, animals n = 3, sprGR cells n = 112, animals n = 5, rPL cells n = 76, animals n = 5, sprPL cells n = 60, animals n = 5, OE cells n = 66, animals n = 5, SP cells n = 174, animals n = 5, PNAm+ cells n = 46, animals n = 5

Supplementary table S2 Comparison of cell dimensions and statistical analysis (*p*-values) of the cytometric data shown in Fig. 2, One-Way-ANOVA and post-hoc pairwise analysis (Tukey-test)

Haemocyte length [µm]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.0110398	-				
SP	0.7975833	0.0001074 ***	-			
rPL	0.4080174	0.8757483	0.0221555 *	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0.2151145	0.9772673	0.0074831	0.999851	0 ***	-
PNAm+	0.9975917	0.0092327 **	0.9928197	0.2488556	0 ***	0.1284124

Haemocyte width [µm]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.9999984	-				
SP	0.7088514	0.5545922	-			
rPL	0.503945	0.5338877	0.0213787 *	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0.2280589	0.2351917	0.0048936 **	0.9992203	0 ***	-
PNAm+	0.9998618	0.9988498	0.9447655	0.4480311	0 ***	0.2191943

Haemocyte length/width

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0 ***	-				
SP	0.976258	0.0000101 ***	-			
rPL	0.9999999	0 ***	0.963466	-		
sprPL	0 ***	0.0062833 **	0 ***	0 ***	-	
OE	0.9997856	0 ***	0.8927594	0.9999716	0 ***	-
PNAm+	0.9999321	0.0000002 ***	0.9409127	0.9999922	0 ***	1

Haemocyte area [µm2]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.9996648	-				
SP	0.9995434	0.9846875	-			
rPL	0.9977688	0.9999938	0.969715	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0.9921326	0.9998405	0.9418144	0.9999994	0 ***	-
PNAm+	0.9999987	0.9993978	0.9999982	0.997557	0 ***	0.9935715

Level of significance indicated by asterisks: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. For sample size, please refer to Supplementary table S1

Supplementary table S3 Comparison of nuclei dimension and statistical analysis (*p*-values) of the data shown in Fig. 2, One-Way-ANOVA and post-hoc pairwise analysis (Tukey-test)

Nucleus length [µm]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.8774779	-				
SP	0.7310479	0.0647721	-			
rPL	0 ***	0 ***	0 ***	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0 ***	0 ***	0 ***	0.0000073 ***	0 ***	-
PNAm+	0.676252	0.9912137	0.0726404	0 ***	0 ***	0.0000002 ***

Nucleus width [µm]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.9999988	-				
SP	0.0016233 **	0.0008057 ***	-			
rPL	0 ***	0 ***	0 ***	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0 ***	0 ***	0 ***	0.0833951	0 ***	-
PNAm+	0.7670549	0.6423261	0.0000905 ***	0.0047555 **	0 ***	0.0000005 ***

Nucleus length/width

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.7104003	-				
SP	0.0000509 ***	0.0051948 **	-			
rPL	0 ***	0.0000021 ***	0.768254	-		
sprPL	0.0004703 ***	0.0298539	0.9996884	0.5241554	-	
OE	0.9707772	0.1490822	0.0000006 ***	0 ***	0.0000092 ***	-
PNAm+	0.9999959	0.7894768	0.0010933	0.0000034 ***	0.0047575 **	0.99811

Nucleus area [µm2]

	GR	sprGR	SP	rPL	sprPL	OE
sprGR	0.9983098	-				
SP	0.7499775	0.3874797	-			
rPL	0 ***	0 ***	0 ***	-		
sprPL	0 ***	0 ***	0 ***	0 ***	-	
OE	0 ***	0 ***	0 ***	0.9611964	0 ***	-
PNAm+	0.8813264	0.9790609	0.2210812	0.000029 ***	0 ***	0.0006936 ***