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Changes induced by two strains of *Vibrio splendidus* in haemocyte subpopulations of *Mya arenaria*, detected by flow cytometry with LysoTracker

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ABSTRACT: Flow-cytometric characterisation of bivalve haemocytes is usually performed by light-scatter profiles based on size and complexity of the cells. Additional means of characterisation such as specific fluorescent dyes are not commonly used to discriminate cell subpopulations in challenged and unchallenged haemocytes. In the present study, we characterise the changes in haemocyte subpopulations of soft-shell clam *Mya arenaria* induced by *in vivo* challenge with 2 strains of *Vibrio splendidus* by using a fluorescent probe. Responses were measured 24 h after infection with either a local wild strain (7SHRW) or a modification (LGP32-GFP) of a strain associated with oyster mortalities in France (LGP32). Changes in haemocyte subpopulations were analysed using flow cytometry based on 2-parameter scatter profiles and lysosomal content reflected by LysoTracker staining. Forward and side-scatter profiles revealed 2 haemocyte subpopulations: hyalinocytes and granulocytes. Granulocytes exhibited significantly higher levels of lysosomal staining ($p < 0.01$). Following infection with LGP32-GFP, both subpopulations merged into a single continuous group and their lysosomal content significantly decreased ($p < 0.05$). Independent modifications after infection were observed in the proportions of subpopulations established by their lysosomal content. While the subpopulation of hyalinocytes had lower levels of lysosomal content after infection, especially with LGP32-GFP ($p < 0.001$), the subpopulation of granulocytes had similar levels of lysosomes after infection with 7SHRW and significantly decreased levels after infection with LGP32-GFP ($p = 0.001$). Our data suggest specific modulation of bivalve responses against pathogenic bacteria that would include degranulation.

KEY WORDS: *Mya arenaria* · *Vibrio splendidus* · Haemocyte subpopulations · Flow cytometry · LysoTracker · Lysosome

INTRODUCTION

Haemocytic ontogeny and typing are undefined aspects of haemocytic characterisation in bivalve molluscs. In the absence of well-defined immunophenotypic markers comparable to those typically employed to study analogous human peripheral blood leucocytes, the characterisation of haemocytic subpopulations in bivalves relies upon morphological features including cell size, complexity or granularity, or functional characteristics including phagocytosis and oxidative burst (Huffman & Tripp 1982, Cajaraville &

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Pal 1995, López et al. 1997, Pipe et al. 1997, Cima et al. 2000, Chang et al. 2005, Zhang et al. 2006, Aladaileh et al. 2007, García-García et al. 2008). Interspecies variability and methodological differences in specimen collection and processing have been considered the principal reasons for the current lack of consensus on the actual number of distinct haemocytic subpopulations that can be discerned morphologically (see reviews in Cheng 1981, 1984, Auffret 1988, Hine 1999). There is general agreement, however, that bivalve haemocytes can readily be classified into at least 2 morphologically distinct groups, namely the granulocytes, which tend to be larger and contain cytoplasmic granules, and hyalinocytes (or agranulocytes), which tend to be smaller and contain fewer or no granules.

The cytoplasmic granules that characterise granulocytes are mostly lysosomes, membrane-bound organelles containing hydrolytic enzymes at low pH (Luzio et al. 2000). In bivalves, haemocyte lysosomes are known to be involved in the intracellular degradation of digestible material and the release of hydrolytic enzymes during phagocytosis in response to infection (reviewed in Cheng 1983). Since their membranes are susceptible to being destabilised by different stressors, this feature has been frequently used as a biomarker to monitor pollution and animal health (reviewed in Moore et al. 2004, 2006).

Since the introduction of flow cytometry to the study of bivalve haemocytes, this tool has gained gradual acceptance due to its practical use and avoidance of subjectivity compared with traditional methods of cell characterisation (Fisher & Ford 1988, Ashton-Alcox et al. 2000). The application of light-scatter profiling has been used to differentiate bivalve haemocyte subpopulations (Ashton-Alcox & Ford 1998, Allam et al. 2002, García-García et al. 2008) and to monitor changes in several immune indicators after bacterial challenges (Allam et al. 2001, 2006, Choquet et al. 2003, Lambert et al. 2003, Allam & Ford 2006, Labreuche et al. 2006). In addition, haemocyte characterisation using fluorescent dyes with affinity to cellular organelles and monoclonal antibodies for specific cellular types conjugated with fluorescent dyes has been used in combination with light-scatter profiles (Renault et al. 2001, Tu et al. 2007).

In soft-shell clams, granulocytes have been successfully discriminated from agranulocytes based on both light-microscopic (Huffman & Tripp 1982) and flow-cytometric analyses (Brousseau et al. 1999, Fournier et al. 2001, 2002). Recently, using flow-cytometric analysis, we reported changes in cell numbers and adhesion of *Mya arenaria* haemocytes infected with 2 strains of *Vibrio splendidus* (Mateo et al. 2009). A shift in the distribution of Manila clam *Ruditapes philippinarum* granulocytes after *in vitro* challenge with *V. tapetis* has been reported using 2-parameter scatter profiles (Allam & Ford 2006). Whether bacterial challenge induces changes in the distribution of haemocyte subpopulations of *M. arenaria* is still unknown.

Given its acidic tropism, the commercially available probe LysoTracker has been used to detect lysosomes in studies of human cells (Haller et al. 1996, Via et al. 1998, Blander & Medzhitov 2004). Here, we report the changes in the distribution of haemocyte subpopulations of *Mya arenaria* induced by *in vivo* challenges with 2 strains of *Vibrio splendidus* using LysoTracker Red. By using this method we supplement light-scatter characterisation of subpopulations of soft-shell clam haemocytes with profiles of cellular lysosomal content.

MATERIALS AND METHODS

Clams. Wild soft-shell clams *Mya arenaria* (approximately 4 to 5 cm in length and 17 g in weight) shipped from the Centre Maricole des Iles-de-la-Madeleine (CEMIM; Gulf of St. Lawrence, Canada) were used, as they are exposed to minimal levels of pollution. Clams were held in 300 l tanks with recirculating synthetic seawater (Instant Ocean®, Aquarium Systems). Throughout the acclimation period clams were fed Spat Formula (Innovative Aquaculture Products) every other day, and water temperature was kept at 16°C and salinity at 30.

Bacteria. *Vibrio splendidus* LGP32 is a strain associated with mortalities in juvenile oysters *Crassostrea gigas* in France (Gay et al. 2004a,b), and we used a modified strain (LGP32-GFP) which has a green fluorescence protein (GFP) gene insertion that confers fluorescence through UV light. *V. splendidus* 7SHRW (GenBank accession no. FJ610758) is a wild strain isolated from sediments from Hillsborough River, Prince Edward Island (Gulf of St. Lawrence, Canada) (Mateo 2006). The identification of this strain as *V. splendidus* was based on the combination of conventional biochemical tests, BIOLOG automated identification and determination of its 16S rDNA sequence, which possesses 98% similarity to the 16S rDNA on chromosome 1 of LGP32 (GenBank accession number FM954972).

Bacterial exponential growth was achieved overnight in trypticase soy broth (TSB; BD-Bacto™) supplemented with 2% NaCl at 16°C. Bacteria were suspended in filtered (0.22 µm) sterile seawater (FSSW) after 2 steps of centrifugation (5000 × *g*, 16°C, 10 min) and rinsing. Bacterial concentration was adjusted to approximately 3.8×10^8 bacteria ml^{-1} in FSSW according to 1 optical density $(OD)_{600\ nm} = 4 \times 10^8$ bacteria ml^{-1} as estimated by flow-cytometry cell counting.

Clam pre-screening and inoculation. In order to avoid using samples from unhealthy or stressed clams, haemolymph was pre-screened through microscopic observation immediately before experiments as previously described (Mateo et al. 2009). Briefly, a drop of haemolymph from each clam was placed on a slide for over 15 min to allow healthy haemocytes to adhere and stretch onto the glass surface. An Axio Imager A1 (Carl Zeiss) light-fluorescent microscope with phase contrast (×400) was used to detect bacteria and assess the percentage of rounded haemocytes estimated by calculating the average measurement from 5 different fields. A threshold of >5% and/or obvious presence of bacteria were used to exclude unfit animals.

In a total of 21 clams, the posterior adductor muscle was injected with 200 µl of either FSSW (control) or 1 of the 2 bacterial suspensions, containing approximately 4.5×10^6 bacteria g^{-1} of clam. To assure that the bacterial suspension was retained, clams were kept out of the water for 1 h after injection before transferring them to containers with non-circulating artificial seawater at 16°C.

Twenty-four hours after infection, haemolymph was withdrawn from the posterior adductor muscle of each clam with a 3 ml syringe fitted with a 25-gauge needle containing 200 µl of anti-aggregate Alsever's solution (Sigma). From each clam an aliquot of 400 µl of haemolymph was collected and screened through an 80 µm mesh to avoid large particles, and immediately placed on ice to prevent formation of haemocyte aggregates.

Morphological profiling. For the analysis of haemocyte subpopulations, 400 µl haemolymph samples were treated with LysoTracker™ Red (Invitrogen-Molecular Probes), a fluorescent probe that stains lysosomes (Fig. 1). A working solution of LysoTracker was prepared by diluting the stock solution 1:1000 in phosphate-buffered saline (PBS) supplemented with 2% NaCl, and added to 400 µl haemolymph samples at a ratio of 1:20. Samples were incubated on ice and in darkness for 2 h, to allow adequate staining prior to analysis in a FACSaria cell sorter (BD Biosciences). PE-Texas Red channel (600 to 620 nm) was used for LysoTracker detection. Excitation was performed with a blue laser (488 nm) and the detector PMT voltage was adjusted so that unstained cells appeared in the first decade (i.e. relative fluorescence < 300). The distribution of haemocytes was characterised according to

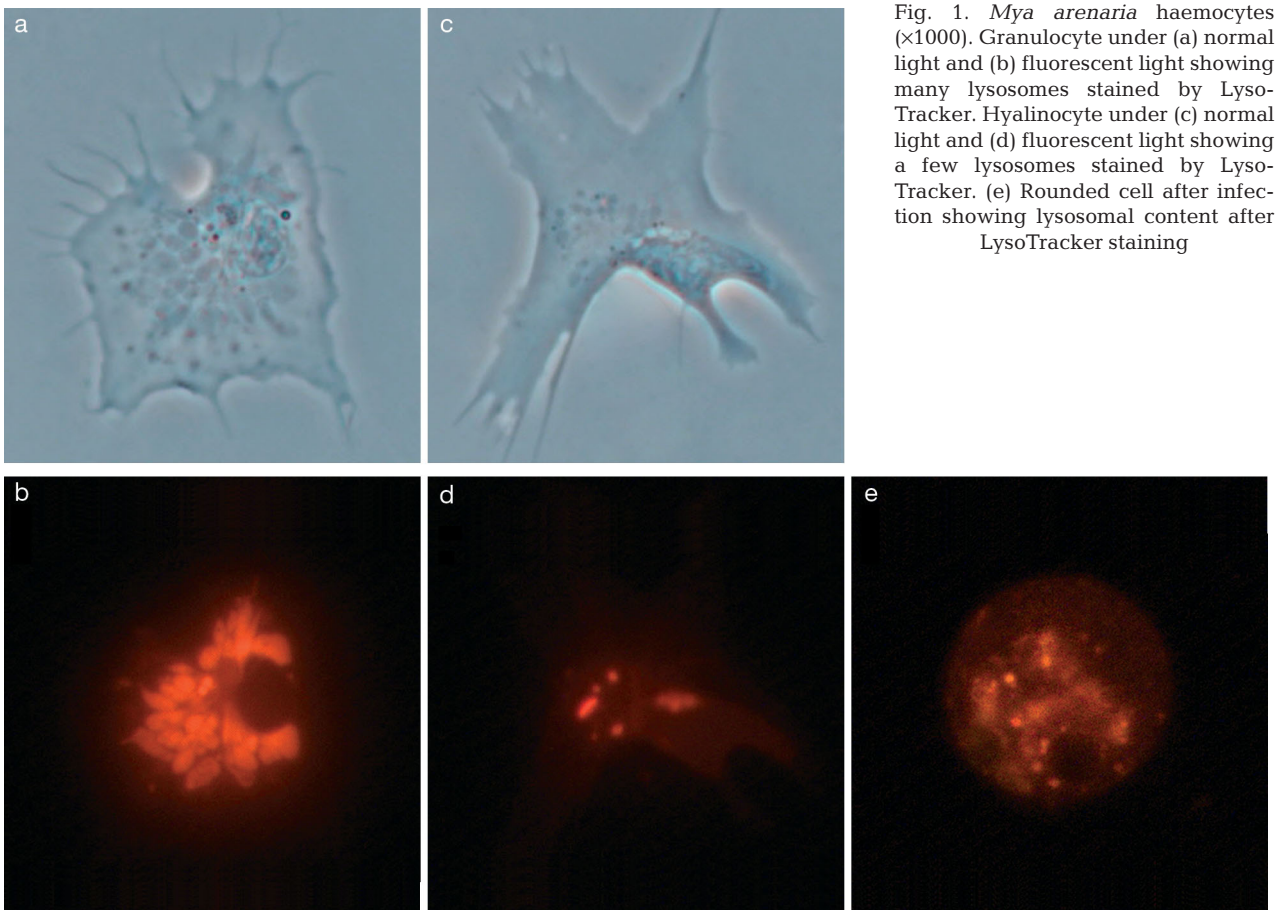

Fig. 1. *Mya arenaria* haemocytes (×1000). Granulocyte under (a) normal light and (b) fluorescent light showing many lysosomes stained by LysoTracker. Hyalinocyte under (c) normal light and (d) fluorescent light showing a few lysosomes stained by LysoTracker. (e) Rounded cell after infection showing lysosomal content after LysoTracker staining

their size and complexity (dependent on the presence of granules and organelles) using 2-parameter scatter profiling: side scatter versus forward scatter. Arbitrary gating was drawn around distinct subpopulations of cells that were readily discernible in healthy specimens. The intensity of fluorescence emitted by LysoTracker was simultaneously measured in a separate fluorescence channel from each gate both before and after infection.

Haemocytes from control and LGP32-GFP-infected clams belonging to each of the subpopulations established by combining the light-scatter and the LysoTracker-staining profiles were physically sorted and immediately fixed with 6% formalin (prepared with FSSW) for observation by light-fluorescent microscopy under a rhodamine filter. Images were obtained using an Axio Cam imaging system.

Statistical analysis. Statistical analysis was performed using MINITAB 15.1.0.0 statistical software. The differences in fluorescence intensity among haemocyte subpopulations were tested using 1-way general linear ANOVA followed by Bonferroni pairwise comparison to $\log_{10}$-transformed data. The differences in the proportions of haemocytic subpopulations were also tested using ANOVA and Bonferroni pairwise comparison of the arcsine of the square-root-transformed data. When a normal distribution was not achieved, the Kruskal-Wallis nonparametric model was applied followed by the Mann-Whitney test for pairwise comparisons. A statistical significance level of 0.05 was used for differences in all tests.

RESULTS

Forward versus side-scatter profiles revealed that healthy *Mya arenaria* haemocytes were distributed into 2 discernible subpopulations: 1 group (69.1 ± 23.1% SE of the haemocytes) composed of larger and more complex cells, considered to be granulocytes, and another group (30.9 ± 3.1% of the haemocytes) composed of smaller and less complex cells, considered to be hyalinocytes or agranulocytes (Figs. 2 & 3a). After infection with 7SHRW, the latter subpopulation in the scatter profile became less discernible (Fig. 3b), while with LGP32-GFP infection, both subpopulations appeared to coalesce into a single continuous group (Fig. 3c). When the arbitrary gates established for healthy clam haemocyte subpopulations were maintained, the proportions of these 2 subpopulations did not change significantly after infection with either strain ($p > 0.05$, Figs. 2 & 3).

Comparison of the median of fluorescent intensity values of LysoTracker staining showed that in control clams, granulocytes exhibited significantly more lysosomal staining than did hyalinocytes ($p < 0.01$) (Fig. 4). A similar tendency was found after infection with 7SHRW ($p < 0.001$) and LGP32-GFP ($p < 0.001$). However, the median fluorescence intensity revealed that the lysosomal staining in both haemocyte subpopulations in LGP32-GFP-infected clams was significantly lower than those from control clams ($p < 0.01$ for granulocytes, $p < 0.0001$ for hyalinocytes) and from 7SHRW-infected clams ($p < 0.001$ for granulocytes, $p = 0.0001$ for hyalinocytes) (Fig. 4).

When each haemocyte subpopulation was further subdivided according to lysosomal content (herein defined as the relative amount of LysoTracker fluorescence observed) into 'low' and 'high' (Fig. 5), the proportions among groups significantly changed after infection, for both hyalinocytes ($p < 0.001$) and granulocytes ($p = 0.001$) (Fig. 6). Among hyalinocytes, the proportion of cells with high lysosomal content significantly decreased from 85.3 ± 2.2% in the control clams to 69.4 ± 4.7% in the 7SHRW-infected clams and to 21.1 ± 5.4% in the LGP32-GFP-infected clams (Fig. 6a). Among granulocytes, the proportion of cells with high lysosomal content varied little between the control and the 7SHRW-infected groups, each having >80% of cells with high lysosomal content (87.8 ± 0.4% and 86.6 ± 1.8%, respectively) and <15% of cells with low lysosomal content (12.2 ± 0.4% and 13.4 ± 1.8%, respectively). These proportions, however, were somewhat inversed in clams infected with LGP32-GFP, with 60.2 ± 10.6% of cells having low and 39.8 ± 10.6% of cells having high lysosomal content (Fig. 6b).

Microscopic observation of the sorted cells belonging to each of the 4 subpopulations established by the 2-scatter and LysoTracker staining profiles re-

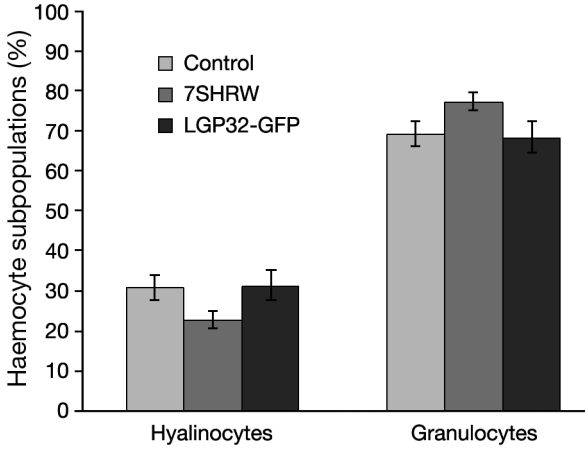

Fig. 2. *Mya arenaria* haemocytes. Proportions (±SE) of hyalinocytes and granulocytes from clams 24 h after injection with filtered sterile seawater (FSSW; control), *Vibrio splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Differences among the treatments were not significant ($p > 0.05$)

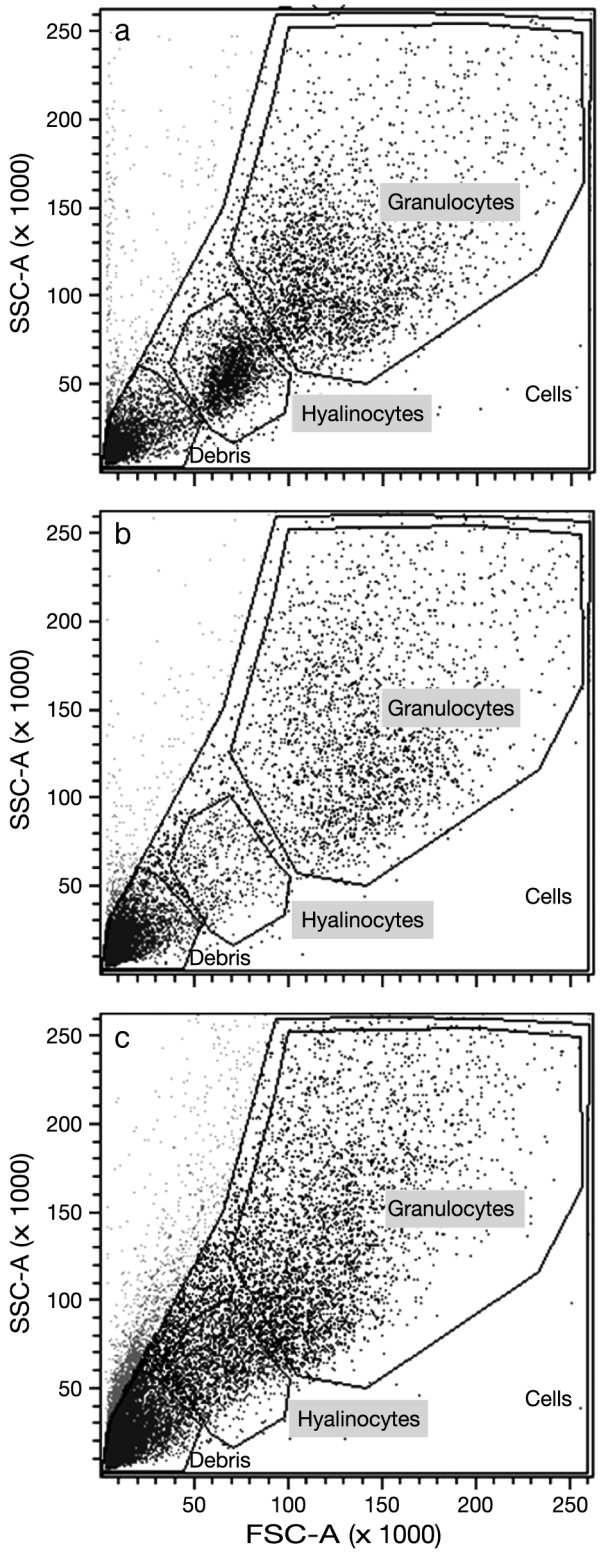

Fig. 3. *Mya arenaria* haemocyte distribution profile. Forward-scatter (FSC) and side-scatter (SSC) plot profiles of granulocytes and hyalinocytes from clams 24 h after injection with (a) filtered sterile seawater (FSSW), (b) *Vibrio splendidus* 7SHRW or (c) *V. splendidus* LGP32-GFP

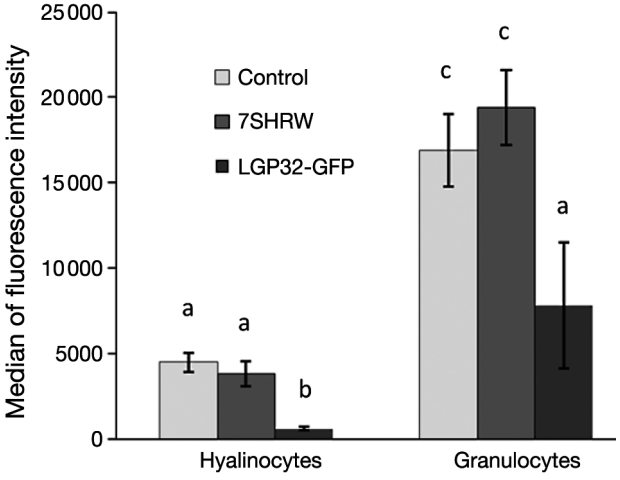

Fig. 4. *Mya arenaria haemocytes*. Median (±SE) fluorescence intensity of LysoTracker retention in granulocytes and hyalinocytes from clams 24 h after injection with filtered sterile seawater (FSSW; control), *Vibrio splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Letters show statistical equivalence (same letters) or difference at $p < 0.05$ (different letters) between pairs of haemocyte subpopulations and treatments

vealed cells of different sizes and cytoplasm content (Fig. 7). Hyalinocytes had a diameter of approximately 5 to 7 µm (Fig. 7a,b,e,f) whereas in granulocytes it was around 9 to 13 µm (Fig. 7c,d,g,h). Haemocytes sorted from the 'high' LysoTracker staining subpopulation (Fig. 7b,d,f,h) appeared to have a higher granularity compared with those with 'low' staining (Fig. 7a,c,e,g). To some extent, the cytoplasm content of the control haemocytes appeared to be homogeneously distributed, while in LGP32-GFP-infected cells it appeared to be localised towards one side of the cell and the nucleus was often indistinguishable.

DISCUSSION

Flow cytometry is a useful technique for the characterisation of haemocyte subpopulations in bivalves. Analyses using these methods are mostly based on 2-side scatter profiles that delineate subpopulations according to cell size and complexity. Additional fluorescent dyes can be used to reveal complementary information about cellular components for a more complete characterisation. In the present study, the use of LysoTracker, as an indicator of lysosomal content, in addition to light-scatter profiles, revealed different haemocyte subpopulations in *Mya arenaria*. These subpopulations were shown to undergo changes in proportions that suggest interesting cellular processes induced by 2 strains of *Vibrio splendidus*, a local wild

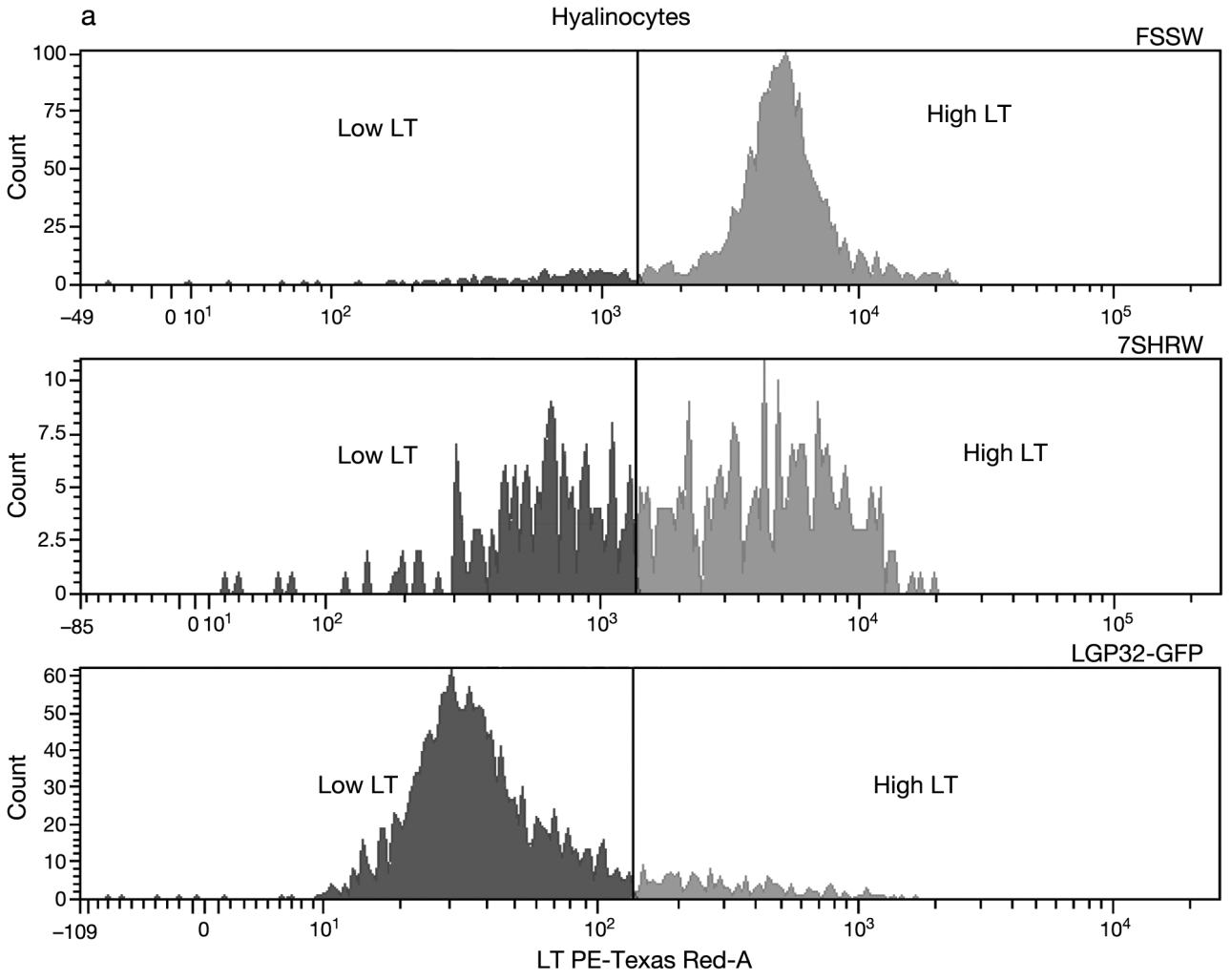

Fig. 5. *Mya arenaria* haemocytes with LysoTracker. Median fluorescence intensity of LysoTracker (LT) retention (arbitrarily established as 'high' and 'low') in (a) hyalinocytes and (b) granulocytes from clams 24 h after injection with filtered sterile seawater (FSSW; top), *Vibrio splendidus* 7SHRW (middle) or *V. splendidus* LGP32-GFP (bottom)

strain and a strain associated with oyster mortalities in France.

Through the flow-cytometry scatter profiles (forward and side-scatter) we observed 2 discernible subpopulations of haemocytes in *Mya arenaria*: a discrete subpopulation of smaller and less complex cells and another, more dispersed, subpopulation of larger and more complex cells. We think that the former subpopulation was composed of hyalinocytes and/or agranulocytes (with no or few cytoplasmic granules) and the latter, granulocytes. This latter subpopulation might include what others have classified as small and large granulocytes (reviewed in Cheng 1981). Previously, using flow-cytometric toxicological studies of *M. arenaria* haemocytes, 2 discernible subpopulations using 2-scatter profiles were also noticed (Brousseau et al. 1999, Fournier et al. 2001, 2002).

Prior reports involving flow-cytometric light-scatter profiling revealed that haemocytes from a number of bivalve species can typically be classified into 2, 3 or 4 subpopulations. Haemocytes from clams *Ruditapes philippinarum*, *R. decussatus* and *Mercenaria mercenaria* have been classified into 2 subpopulations: hyalinocytes and granulocytes, located in the lower and higher channels of both light-scatter axes, respectively (Allam et al. 2001, 2002, 2006, Allam & Ford 2006). In the scallop *Chlamys farreri*, 2 haemocyte types have been identified: granulocytes and hyalinocytes (Xing et al. 2002). The hard clam *Meretrix lusoria* has been reported to have 3 subpopulations: hyalinocytes and small and large granulocytes (Tu et al. 2007). In mussels *Mytilus galloprovincialis*, 3 subpopulations (hyalinocytes and small and large granulocytes) (Parisi et al. 2008) and 4 subpopulations (large granu-

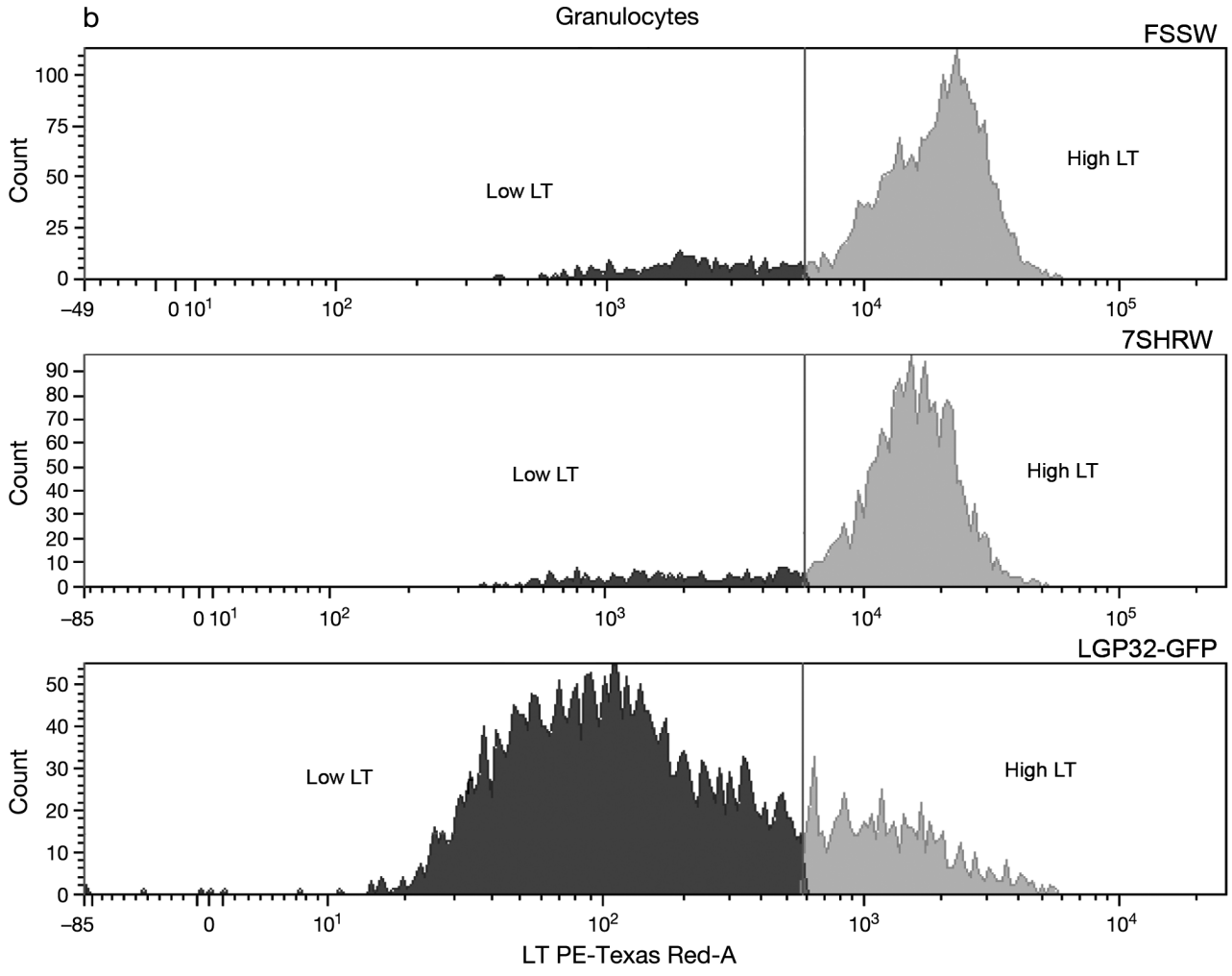

Fig. 5 (continued)

locytes, large semigranulocytes, smaller granulocytes and small agranulocytes or hyalinocytes) (García-García et al. 2008) have been considered. Oysters *Crassostrea virginica* and *C. gigas* were shown to possess 3 haemocyte subpopulations, although they were regarded slightly differently by different researchers (Ashton-Alcox & Ford 1998, Allam et al. 2002, Lambert et al. 2003, Goedken & De Guise 2004). In the Sydney rock oyster *Saccostrea glomerata*, up to 4 subpopulations were distinguished, although hyalinocytes and granulocytes were the 2 most abundant phenotypes (Aladaileh et al. 2007).

Following infection with *Vibrio splendidus* 7SHRW, the proportion of hyalinocytes slightly decreased (Fig. 2) and became less discernible in the scatter profile (Fig. 3b), while with the strain LGP32-GFP, changes were more striking, as both subpopulations merged into a single continuous group (Fig. 3c). Drastic changes in haemocyte distribution according to size and granularity have been previously noticed by Allam & Ford (2006). They observed a clear shift of granular cells towards the agranular cell population, resulting in unimodal distribution of haemocytes from clams *Ruditapes philippinarum* and *Mercenaria mercenaria* after *in vitro* exposure to *V. tapetis* and *V. splendidus*.

We supplemented our light-scatter profiling data with LysoTracker staining in order to provide a measure of lysosome content. LysoTracker is a fluorescent probe that accumulates in acidic compartments (Freundt et al. 2007) and has been used previously in studies of human-cell lysosomes (Haller et al. 1996, Via et al. 1998, Blander & Medzhitov 2004). This approach, applied for the first time to bivalve haemocytes in the present study, revealed independent responses in haemocyte subpopulations (Figs. 4, 5 & 6). LysoTracker staining showed that granulocytes from the control clams contained significantly more lysosomes than the hyalinocytes. This is in agreement with microscopic observations, and indirectly with enzyme studies, which have shown that granulocytes have abundant

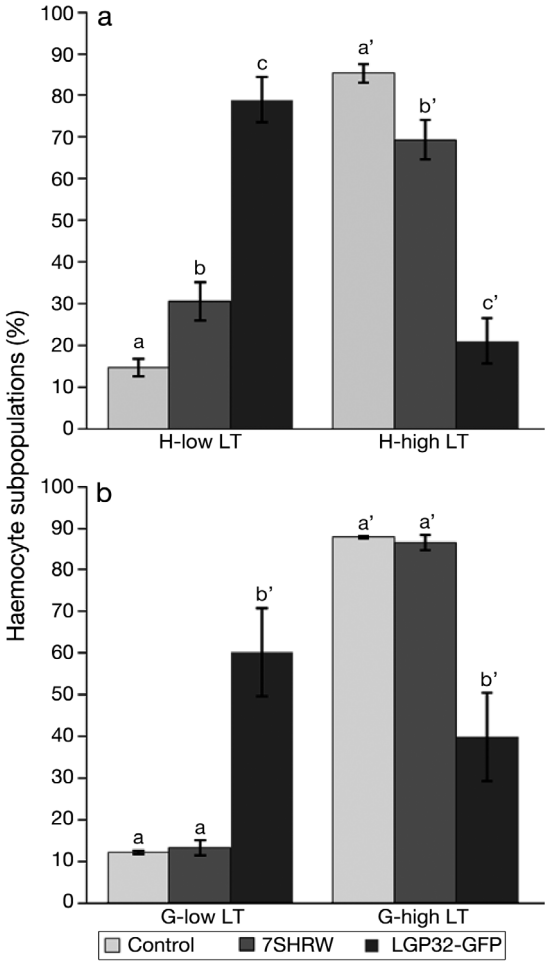

Fig. 6. *Mya arenaria* haemocyte subpopulations. Proportion (±SE) that are (a) hyalinocytes (H) or (b) granulocytes (G) according to LysoTracker (LT; 'high' or 'low') staining 24 h after injection of clams with filtered sterile seawater (FSSW; control), *Vibrio splendidus* 7SHRW or *V. splendidus* LGP32-GFP (n = 21). Letters show statistical equivalence (same letters) or difference at $p < 0.05$ (different letters) among treatments

lysosomes (Pipe 1990, Cajaraville & Pal 1995, Cima et al. 2000, Matozzo et al. 2007).

When challenged bacterially, hyalinocytes had lower levels of lysosomal content, especially after infection with LGP32-GFP (Figs. 4, 5a & 6a), whereas granulocytes had similar levels of lysosomes after infection with 7SHRW and significantly decreased levels after infection with LGP32-GFP (Figs. 4, 5b & 6b). Lysosomes are cellular organelles that contain hydrolytic enzymes (Luzio et al. 2000), including lysozymes, that are involved in intracellular degradation and host defence (Olsen et al. 2003). Many lysosomes have secretory functions (Holt et al. 2006). Similar to mammalian macrophages, in several bivalves including *Mya arenaria*, lysosomes release their content upon infection during degranulation of actively phagocytosing cells (Cheng & Rodrick 1974, Rodrick 1979; reviewed in Cheng 1983, Chu 1988). Lysosomal degranulation may be one possible mechanism accounting for the decrease of lysosomal content, expressed as loss of fluorescent intensity (Figs. 4 & 5) and change in proportions (Fig. 6) in haemocytes from *Vibrio splendidus* LGP32-GFP-infected clams.

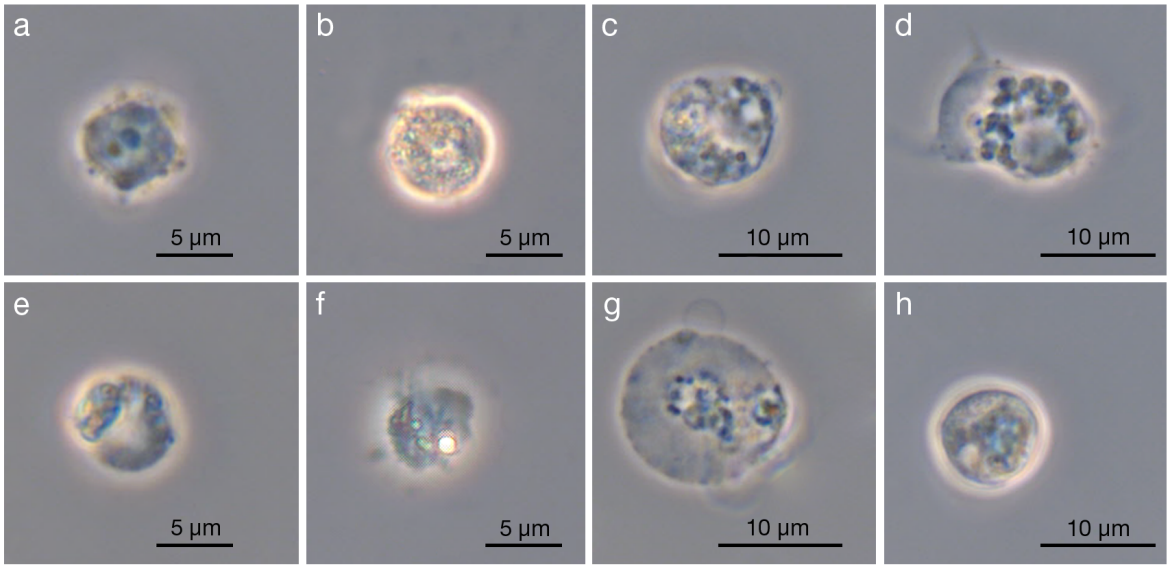

Fig. 7. *Mya arenaria* haemocytes. Phase-contrast images of immediately fixed haemocytes from each subpopulation. Control group: hyalinocytes with (a) 'low' and (b) 'high' LysoTracker staining; granulocytes with (c) 'low' and (d) 'high' LysoTracker staining. *Vibrio splendidus* LGP32-GFP-infected group: hyalinocytes with (e) 'low' and (f) 'high' LysoTracker staining; granulocytes with (g) 'low' and (h) 'high' LysoTracker staining

The decreased responsiveness to *V. splendidus* 7SHRW insult could, by extension, be attributable to a decreased capacity to activate degranulation in *Mya arenaria* haemocytes.

Considering that 7SHRW is a wild endemic strain isolated from sediments from an area relatively close to the source area of our clams, where there is no history of bacterial infections, this strain may be recognised by the haemocyte's pattern-recognition receptors as a non-threat, non-self particle. On the other hand, LGP32 is a non-native strain that has been associated with mortalities of juvenile Pacific oysters *Crassostrea gigas* in Europe (Gay et al. 2004a,b). Moreover, it has been found that this strain possesses the pathogenic factor vsm, a metalloprotease recently demonstrated to be the most important toxicity factor in the extracellular products of LGP32 (Binesse et al. 2008). It is likely that this pathogenic factor is associated with the significant degranulation of *Mya arenaria* haemocytes in the present study. The specific response of *M. arenaria* haemocytes to the strain LGP32-GFP is in accordance with our previous findings that this strain induces significant changes in haemocyte structure, number and adhesion on this host species, whereas changes induced by 7SHRW are minor or nonexistent (Mateo et al. 2009).

The decrease in lysosomal content in hyalinocytes (Figs. 4 & 5b) or the increase of hyalinocytes with low levels of lysosomal content (Figs. 5a & 6a) might not only be due to degranulation but possibly due to the increased presence of precursor haemocytes that have not yet developed cytoplasmic granules. They might be released prematurely to fight the infection. A similar phenomenon, known as 'left shift', occurs in mammalian leucocytes upon inflammation and septic shock (Opdenakker 2001). Undifferentiated and small stem cells have been described as blast-like cells or haemoblasts, a subtype of agranular cells with a high nucleus:cytoplasm ratio and which lack organelles (Hine 1999, Cima et al. 2000, Chang et al. 2005, Aladaileh et al. 2007, Matozzo et al. 2008). Further research is, however, required to confirm the nature of the abundant smaller and less complex cells we found after *Vibrio splendidus* LGP32-GFP challenge.

In conclusion, we found that responses of haemocytic subpopulations are not only specific to the pathogen strain, but are modulated independently of each other and possibly through independent cellular mechanisms. These changes in haemocytic subpopulations were monitored by using LysoTracker as an indicator of the lysosomal content, and more striking responses were found after infection with *Vibrio splendidus* LGP32-GFP. Functional studies are needed to confirm the activation of degranulation and the suspected release of immature haemocytes.

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