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Haemogram of *Littorina littorea*

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The haemogram (blood cells concentration, number and proportions of haemocyte morphotypes) of the common periwinkle, *Littorina littorea*, was studied in terms of its individual variations and seasonal dynamics. No granular blood cells were found. Three morphotypes of hyalinocytes were observed in the haemolymph of *L. littorea* and are described at both light and electron microscopy levels: juvenile round cells, intermediate cells and large mature haemocytes. The nucleoplasmic ratio, nucleus shape and location, number and size of glycogen deposits were the main characteristics used to discriminate between the haemocyte morphotypes. The enzyme cytochemistry and lectin binding studies did not identify additional haemocyte morphotypes. The experiments on BrdU incorporation showed that haemocyte population renewal occurred due to the proliferation of only one cell type, namely blast cells, which were morphologically similar to juvenile round cells. The three-month dynamics of the periwinkle haemogram and haemoblast proliferative activity are described. The blood cell concentration reached maximum values twice per summer season—in June and August, with the haemocyte proliferation activity being the highest in June. The percentage of mature haemocytes increased during the season, simultaneously intermediate cells demonstrated a decrease in number by the end of summer, with the population of juvenile cells being nearby constant. The method of haemogram analysis is positioned as a powerful technique having good potential in eco-toxicological, parasitological and immunobiological studies.

INTRODUCTION

The internal defence system of the Mollusca relies upon two types of immunity: humoral and cell-mediated. However, the dominant effectors in both processes of invader killing and elimination of invaders in mollusc are the circulating haemolymph cells (haemocytes). Despite the fact that many studies have been undertaken on the structure and function of gastropod blood cells, there is still a considerable amount of research required to understand immune function in this important class of molluscs. In particular, there have been very few comparative morphological and ultrastructural studies on the cellular blood components of the major gastropod taxa, Prosobranchia. Among the Gastropoda, intensive immunobiological investigations have only been carried out for the basommatophoran pulmonates (genera *Biomphalaria, Bulinus, Lymnaea*) (Bayne et al., 2001). Only two prosobranch snails, *Viviparus ater* (Viviparidae) and *Cerithidea californica* (Potamididae) have been studied in terms of their haemocyte types (Franchini & Ottaviani, 1990; Yoshino, 1976).

The common periwinkle, *Littorina littorea* L. (Prosobranchia: Littorinidae), is a marine intertidal prosobranch mollusc, widespread and numerous on rocky coastlines around northern Europe. This species fulfils many of the criteria required for consideration as eco-toxicological bioindicators and has shown great potential as a suitable species for investigating both fundamental aspects of prosobranch immunity and also the host-parasite relationships which occur between these snails and trematodes. The common periwinkle is an ideal model organism due to the current detailed knowledge of anatomy and biology of the species (Fretter & Graham, 1994), the existing data on physiologically relevant parameters (De Lange & Minnen, 1998; Larade & Storey, 2004) and the well studied fauna of parasites, specifically trematodes, which infect it (Curtis, 2002). In the present study, we introduce the common periwinkle into immunobiological research practice and investigate the main parameters of the haemocyte population—blood cell concentration, number and relative percentage of haemocyte morphotypes and their histogenetic relationships. The first two characteristics are referred to as the haemogram.

MATERIALS AND METHODS

Molluscs and haemolymph sampling

Common periwinkles, *Littorina littorea*, 6–13 years old, were collected during low tide in Kruglaya bay of the Chupa inlet, (Kandalaksha Bay of the White Sea). The animals were maintained in cages until use and given adequate food supplies (fucoid sea grass). Before the experiments were started, all the snails were examined for trematode infections—only individuals that did not shed cercaria were chosen for the experiments. In addition, after haemolymph sampling each snail was dissected and its soft tissues carefully examined under a dissection microscope for the presence of trematode parthenitae, any infected individuals were excluded from the analysis. Age of the snails was determined by counting winter-grade lines on the shell. Prior to haemolymph collection the animals were placed individually in small closed jars

Table 1. *Lectins used to study haemocyte surface.*

Source	Abbreviation	Sugar specificity
Canavalia ensiformis	ConA	α Mannose, α Glucose
Bandeiraea simplicifolia	BSL	α Galactose, α N-AcetylGalactosamine
Helix pomatia	HPA	N-AcetylGalactosamine
Triticum vulgaris	WGA	(N-AcetylGlucosamine)$_2$, Sialyc acid
Wisteria floribunda	WFL	N-AcetylGalactosamine

filled with seawater and maintained under anoxic conditions for several hours in order to evoke analgesia. Haemolymph was taken by inserting a 25-gauge needle into the buccal sinus of the relaxed snails, the haemolymph sample was held on ice until use and for no longer then 10 min. Haemolymph samples from individual molluscs were used in all experiments.

Haemocyte morphology

A drop of freshly collected haemolymph was transferred to untreated or *poly-L*-lysine coated glass slides or to plastic Petri dishes and left for cell attachment and spreading in a humid chamber for 30 min. After fixation, with 4% paraformaldehyde in seawater for 30 min, the slides were rinsed with filtered seawater of natural salinity (FSW; 24) and a coverslip was applied. The study of cell morphology was carried out by phase-contrast microscopy. To examine non-spreading cells haemolymph was collected directly into tubes containing the fixative. Both spread and suspension haemocytes were stained with Mayer's haemotoxylin or Giemsa–Romanovsky dye. After staining, the cells were rinsed (in the case of cell suspensions—several centrifuge passages), embedded in 50% glycerol and examined using light microscopy. For the detection of peroxidase activity, cells were fixed with 4% paraformaldehyde for 10 min and stained for another 10 min using 1 mg/ml diaminobenzidine (DAB, DAKO) and 0.02% H_2O_2 in 50 mM Tris buffer, pH 7.5. To demonstrate acid phosphatase activity, haemocyte monolayers were fixed with a mixture of equal volumes of 4% paraformaldehyde and 96% ethanol for 10 min and then incubated for 2 h at room temperature in a solution containing the following two components: solution A (5 mg naphtol-AS-phosphate previously dissolved in 500 μl dimethylformamide mixed with 40 ml of ice-cold acetate buffer pH 5.2) and solution B (8 drops of 4% $NaNO_2$ mixed with equal amount of pararosanilin). Positive sites for acid phophatase activity stained red. The cell nuclei were counterstained with Mayer's haemotoxylin. To investigate acid phospatase activity during phagocytosis, haemocyte monolayers were incubated with a suspension of zymosan A (Sigma) particles for 40 min, after this the monolayers were rinsed with seawater and treated as described above.

To demonstrate glycogen deposits inside haemocytes, a periodic acid–Schiff (PAS) reaction was performed: cells were fixed with 4% paraformaldehyde, mounted on organosilan (Penta-Sever, St Petersburg)-coated glass slides and incubated sequentially (10 min) in 0.1% periodic acid and Schiff's reagent. Glycogen stained dark magenta. Finally the cells were counterstained in Mayer's haemotoxylin and mounted in glycerol.

Lectin binding

To prepare cell suspensions for lectin staining, equal volumes of freshly collected haemolymph and 4% paraformaldehyde were mixed and left for one hour at room temperature. The lectins (Sigma) investigated are listed in Table 1. The cells were rinsed twice with Tris buffered saline (TBS; 50 mM Tris-HCl, pH 8.0, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 36 mM NaCl and 2 mM KCl) containing 200 mM glycine and incubated for 1 h in FITC-labelled lectin diluted up to 25 μg/ml in TBS. Control samples were incubated in TBS in the presence of the specific sugar (200 mM). Following the lectin incubation, the haemocytes were washed twice with TBS and mounted in anti-fading medium (DAKO).

Electron microscopy

The haemocytes were fixed for 1 h in ice-cold fixative comprising 1.5% glutaraldehyde and 1% formaldehyde in 0.2 M cacodylate buffer (pH 7.5), osmolality of the fixative was adjusted to 800mOsm by the addition of sucrose and sodium chloride. After fixation the cells were washed twice with cacodylate buffer and postfixed in 2% osmium tetroxide in 0.2 M cacodylate buffer for 1 h at 4°C. The haemocytes were then dehydrated through an ethanol series and embedded in Spurr's resin (Sigma). Ultrathin sections were counterstained with saturated aqueous uranyl acetate and Reynolds' lead citrate and observed in a Leo EM 910 electron microscope.

Proliferative activity

Six nine-year old snails with shell height of about 26–29 mm were used for the experiments. Each snail was injected in the foot muscle with 150 μl of FSW containing 1 mg/ml 5-bromo-2′-deoxyuridine (BrdU) and 5-fluoro-2′-deoxyuridine (Sigma) at the same concentration. After 12 h, haemolymph was collected into ice-cold 70% ethanol and fixed for one hour on ice. Several samples were divided into two aliquots, one of them being used as a negative control. The cells were settled by centrifugation at 360*g* for 10 min, washed once with phosphate buffered saline (PBS) and resuspended in 2N HCl containing 0.5% Triton X-100 to denature DNA and permeabilize the cell membranes. After incubation in HCl/Triton X-100 solution for 30 min at 37°C, haemocytes were washed sequentially, once with 0.1 M borax solution and twice with PBS and incubated for 30 min at 37°C in 1% milk solution in PBS, to block possible non-specific binding of antibodies. Monoclonal anti-BrdU antibodies (BU-33; Sigma), diluted 1:500 in 0.5% milk in PBS, were applied for 5 h at 37°C. Control samples were not treated with the primary antibodies. The haemocytes were then washed with PBS and treated with peroxidase-conjugated secondary antibodies (1:100; goat-anti-mouse, Sigma) for one hour at 37°C. After this step, the cells were washed twice with PBS, once with 50 mM Tris-buffer pH 7.5,

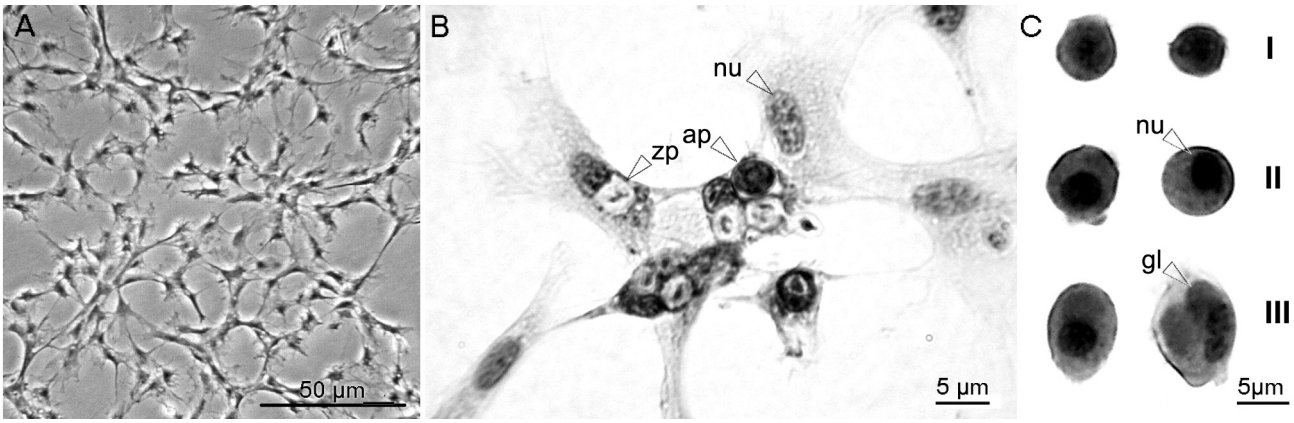

Figure 1. Haemocytes of *Littorina littorea*; (A) monolayer (phase-contrast); (B) monolayer, acid phosphotase reaction in phagocytic cells; and (C) suspension, PAS reaction. ap, acid phosphatase positive phagosome; nu, nucleus; zp, zimozan particle; gl, glycogen; I, II, III, cell morphotypes.

stained for 10 min in Tris buffer containing 1 mg/ml DAB (DAKO) and 0.02% H_2O_2. Finally, haemocytes were rinsed in distilled water to stop the staining reaction and prepared as described above for light-microscopy. The BrdU incorporation was expressed as a BrdU labelling index (BrdULI; a percentage of BrdU-immunopositive cells counted per 400 haemocytes). During the summer of 2003, mean BrdULI values were obtained from three snail batches (10–15 uninfected individuals) freshly gathered at one-month intervals.

Haemogram monitoring

During the summer of 2003, haemocyte population parameters were monitored at two weeks intervals, by sampling haemolymph from about 30 freshly field-gathered uninfected snails. After fixation in suspension, the concentration of circulating blood cells was estimated using a Goryaev's haemocytometer. Cell suspensions were stained with Mayer's haemotoxylin and treated as described above for light-microscopy analysis. The prevalence of the three haemocyte subpopulations (see Results) was estimated by making counts of 400 cells in several fields of view.

Statistical notes

All statistical tests dealt with arcsin-transformed individual percentage estimates. Statistical differences were estimated using two-tailed *t*-test for independent measurements. On figures re-transformed means and 95% confidence limits are shown.

RESULTS

Haemocyte morphology

Freshly sampled *Littorina littorea* haemocytes tended to aggregate avidly, forming clumps of various sizes. However, dilution of the haemolymph with FSW reduced cell clumping and was therefore used to obtain good haemocyte monolayers. The blood cells spread very well on plastic surfaces but less well on glass. Treatment of the glass slides with *poly-L*-lysine did not influence the process of spreading. Phase-contrast microscopic analysis revealed that the dominating cell morphotype was the spreading haemocytes. A small number of round cells and intermediate stages between these and spreading cells were, however, also observed. In monolayers, the cells had an irregular shape and numerous pseudopodia; the cytoplasm was abundant and did not contain any inclusions (Figure 1A).

In suspensions, the haemocytes of *L. littorea* were generally spherical, rarely elongate with short flexuous filopodia. Staining of both suspensions and monolayers with a range of cytological dyes did not reveal any cytoplasmic granules in the cells. Acid phosphatase activity was strictly associated with phagosomes and was only found in haemocytes loaded with zymozan particles (Figure 1B). A positive reaction in this assay was observed in about 8% of phagocytic haemocytes. Both naive and phagocytic cells lacked activity for peroxidase. The PAS-reaction demonstrated the presence of glycogen in the cytoplasm of some haemocytes, there was great variation in the amount of glycogen deposits in the cells being observed, from none to prominent, large glycogen 'lakes' (Figure 1C).

As a whole, three cell subpopulations were found in the haemolymph of *L. littorea* by light microscopy (Figure 1C): glycogen less haemocytes (morphotype I); haemocytes with prominent glycogen deposit (morphotype III); and cells possessing intermediate morphology (morphotype II). Similarly, three types of blood cell were identified by electron-microscopy. They also had distinct morphological features, although a single cell morphotype might offer slightly different aspects.

Morphotype I, 5–6 μm in diameter, had centrically located nucleus of regular shape and high nucleoplasmic ratio (Figure 2A). The cytoplasm was rather scarce with few organelles, a round nucleus: some mitochondria, tubules of smooth endoplasmic reticulum (SER), rare vacuoles, few lipid droplets, numerous free ribosomes and several lysosomes.

Morphotype II, (about 6 μm), represented a heterogeneous subpopulation demonstrating a lower nucleoplasmic

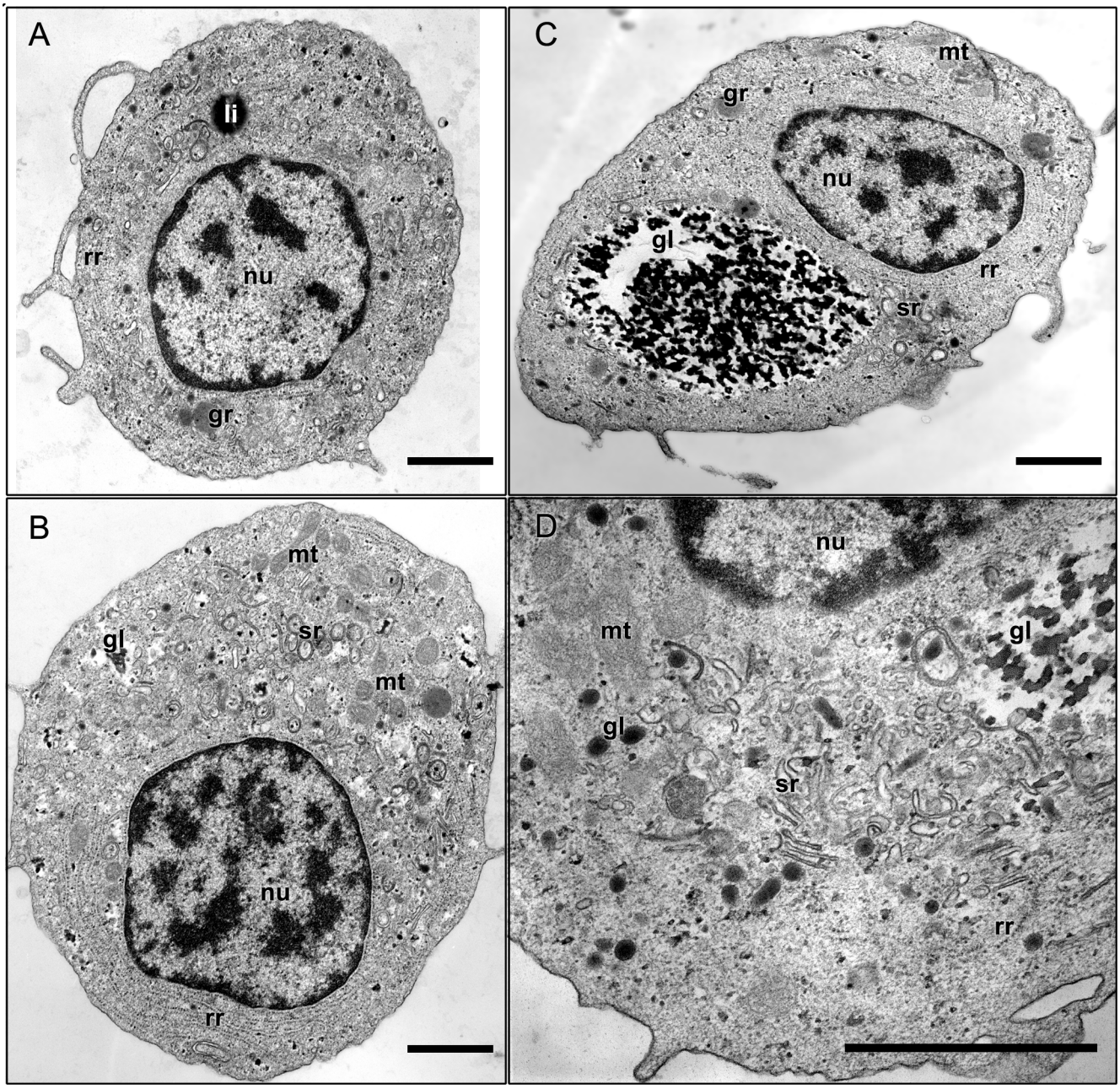

Figure 2. Haemocytes of *Littorina littorea*; (A) juvenile cell; (B) intermediate cell; (C) mature cell; (D) details of mature cell. gl, glycogen; nu, nucleus; mt, mitohondria; sr, smooth reticulum; rr, rough reticulum; li, lipid drop; gr, granule. Scale bar: 1 μm.

ratio compared with the morphotype I haemocytes. These cells had a more or less eccentrically located nucleus and several small glycogen aggregates together with numerous single glycogen particles scattered throughout the cytoplasm (Figure 2B). They also contained mitochondria with a dense matrix, rough endoplasmic reticulum (RER) and developed SER similar to that found in haemocytes of the previous type.

Morphotype III, (7–8 μm), had a small nucleoplasmic ratio; they were asymmetrical with an eccentrically located nucleus (Figure 2C). These cells had a large glycogen 'lake' surrounded by abundant compartments of SER. Cisternae of RER were present around the nucleus and near the narrow cell cortex.

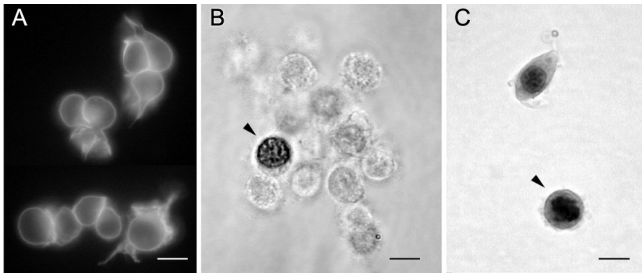

Figure 3. Haemocytes of *Littorina littorea* fixed in suspension. (A) Cells labelled with HPA-FITC; (B) BrdU—positive cell (arrowhead); and (C) mitotic cell (arrowhead). Scale bar: 5 μm.

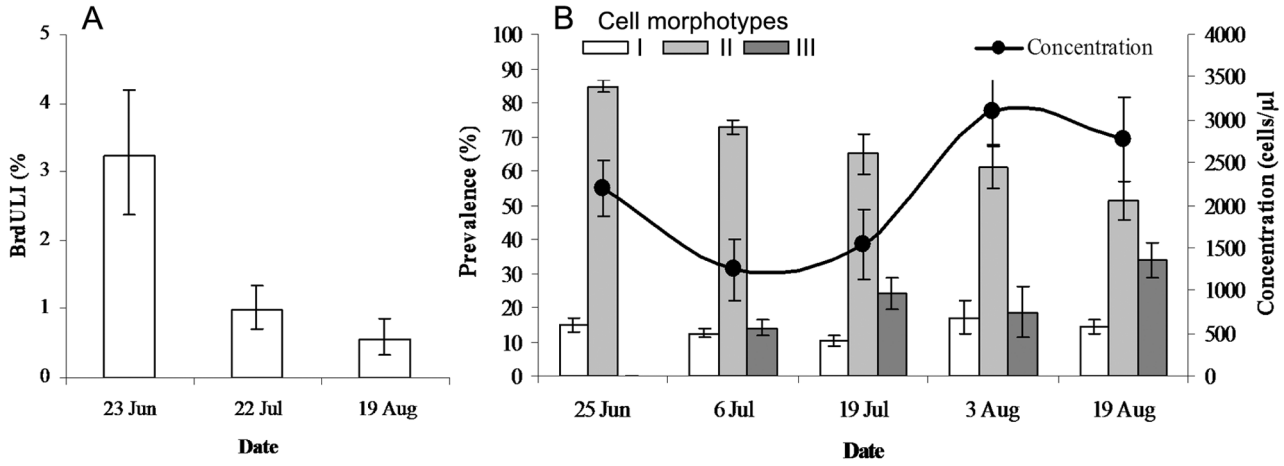

Figure 4. *Littorina littorea* haemogram monitoring; (A) BrdU labelling index (BrdULI); (B) cell concentration and proportion of cell morphotypes I, II, III, in circulation.

Haemocyte surface carbohydrate characterization

Three of the five lectins tested, HPA, ConA and WFL, were shown to bind to the glycocalix of *L. littorea* haemocytes (Figure 3A). The lectin binding was inhibited by the presence of the competing monosaccharides, N-acetylgalactosamine, glucose and mannose, suggesting that these or related sugars might be serving as specific lectin receptors. No distinct haemocyte subpopulations were identified according to the lectin binding; all three lectins demonstrated equal binding to all blood cells. In control samples only minimal background fluorescence with no specific labelling with the lectins was detected.

Haemopoietic activity

The BrDU incorporation was restricted to one cell subpopulation—7 μm haemocytes with high nucleoplasmic ratio and centrically located nucleus with 'structured' chromatin (Figure 3B). Seasonal variability of BrdULI in haemocytes from uninfected snails was detected, the haemopoietic activity being highest (about 3% of *S*-phase cells) in June, when rare mitotic cells were observed in circulation of healthy periwinkles (Figure 3C). The lowest BrdULI values were detected in August (Figure 4A).

Seasonal monitoring of haemogram

This analysis was conducted to investigate temporal fluctuations of periwinkle haemogram parameters during the summer season. Overall, the cell concentration in the *L. littorea* haemolymph ranged from 700 to 4000 cells per ml, with the mean estimates about 2000 cells per ml. However, this range reflects not only individual variation but also a temporal one with two maximum mean values in June and early August (Figure 4B). In the beginning of summer, haemolymph lacked large cells with glycogen 'lakes' (morphotype III). They appeared in July and gradually increased in number, up to about 35%, in late August. Conversely, the proportion of morphotype II cells in the haemolymph decreased from 85% in June to about 50% in August. The subpopulation of morphotype I haemocytes in the circulation of *L. littorea* had significant ($P<0.05$) fluctuations, however, it was relatively constant (mean value about 14%) during the summer.

DISCUSSION

The method used for collection of haemolymph from the snail buccal sinus yields a large volume of haemolymph, free from tissue debris and without contamination of the sample with extravisceral fluids and mucus. These contamination problems may occur during haemolymph sampling procedures requiring shell perforation and destruction of soft tissues, like cardiac puncture which is often used to collect haemolymph from *Biomphalaria glabrata* (Loker & Hertel, 1987) or collection of haemolymph extruded via haemal pore upon foot retraction in *Lymnaea stagnalis* (Amen et al., 1992). The haemolymph sample contamination may result in preliminary haemocyte activation that may sometimes complicate further manipulation of the cells. Using our method we managed to obtain up to 1.5 ml of sterile blood from one snail. It is worth noting that all the snails survived after the haemolymph collection procedure, so individual monitoring of haemogram parameters after different treatments is potentially possible.

Two assays were carried out to classify *Littorina littorea* haemocyte subpopulations in this study. The traditional assay applying to the cells that have attached to glass or plastic slides is informative and very productive for the study of shape and morphology of spreading haemocytes. However, if cells do not attach or only weakly attach to a substrate, or if haemocyte clumping is very pronounced, then this population is not accounted for. To note, from our experience (unpublished) haemocytes of the marine molluscs (*Littorina*, *Buccinum*, *Mya*, *Mytilus*) tended to aggregate much more than their freshwater relatives (*Viviparus*, *Biomphalaria*, *Lymnaea*, *Unio*, *Anodonta*). Moreover, during the process of attaching and plating (15–30 min) important morphological characteristics of blood cells may be dramatically modified due to, for instance, degranulation. The second less common assay examined the haemocytes preserved immediately after haemolymph sampling and conserved the *in situ* state of

the cells and lacked the disadvantages of the previous approach. The practical application of the periwinkle haemogram requires staining of the cells processed in this second way.

The lack of endogenous peroxidase activity in *L. littorea* haemocytes is not a unique phenomenon among gastropod molluscs. Similar negative reactions for peroxidase activity were observed in *Achatina fulica* and *B. glabrata* haemocytes Adema et al., 1992; Matricon-Gondran & Letocart, 1999). On the other hand, activity for acid phosphatase has been documented in three previous studies on other gastropod species: *Lymnaea stagnalis* (Adema et al., 1994), *B. glabrata* (Matricon-Gondran & Letocart, 1999) and *Viviparus ater* (Franchini & Ottaviani, 1990). The *Littorina littorea* haemocytes were positive following phagocytic stimulation, indicating that the acid phosphatase may be present in an inactive form until the cells initiate phagocytosis.

The haemograms of two prosobranch molluscs studied to date *Cerithidea californica* (Yoshino, 1976) and *V. ater* (Ottaviani, 1989; Franchini & Ottaviani, 1990) were shown to include granulocytes as a common cell type. The peculiar characteristic of these cells is the presence of vast amount of granules in their cytoplasm having different morphology, from small, round granules to large, polymorphic ones. Although some rare haemocytes with one or two electron dense granules did occur in *L. littorea* haemolymph it is difficult to refer to them as granulocytes because this term has been designated for haemocytes filled with numerous specific granules, e.g. similar to those of a number of bivalve molluscs (Cajaraville & Pal, 1995; Nakayama et al., 1997; Pipe et al., 1997). Thus, the haemogram of *L. littorea* lacks cells of this type. Differently from *V. ater* (Ottaviani, 1989; Franchini & Ottaviani, 1990) having in a circulation the only haemocyte type—typical granulocytes, periwinkles endowed with a cellular blood system composed of hyalinocytes (agranular cells). Yoshino (Yoshino, 1976) has described both of these cell types in *C. californica* and noted the absence of SER and glycogen deposits in hyaline cells of this species. The hyalinocytes of *L. littorea* have glycogen and well developed SER and in this respect generally resemble the agranular cells of the pulmonate gastropods *Lymnaea stagnalis* (Sminia, 1972) and *B. glabrata* (Matricon-Gondran & Letocart, 1999). However, similar glycogen lakes have also been described in the bivalve mollusc *Scrobicularia plana* (Wootton & Pipe, 2003). This fact may be explained by the rather similar habitat conditions of *Littorina littorea* and *S. plana*—both the molluscs inhabit the inter-tidal zone and must survive rather long periods of low oxygen concentration. So it seems likely that such large stores of fermentable fuels in these animals may be used during anoxia when glycolysis is the only source of ATP.

Haemocyte populations can also be differentiated by their surface glycoconjugates recognized by lectins (Schoenberg & Cheng, 1980; Joky et al., 1983). Both studies by Schoenberg and Cheng (Schoenberg & Cheng, 1980) and Ottaviani (Ottaviani, 1989) have shown binding of concanavalin A to *B. glabrata* and *V. ater* haemocytes, respectively. Moreover, ConA and WGA differentially bound to different types of haemocyte in *B. glabrata* (Joky et al., 1983): 'type IV cells—large haemocytes' labelled by WGA and 'type III cells' bearing receptors for ConA may correspond to the medium-size haemocytes. The haemocytes of *L. littorea* did not show variability in lectin binding between subpopulations and so appear to represent a single uniform population.

As in the case of *Lymnaea stagnalis* (Sminia et al., 1983) the results of ultrastructural investigations corroborate the light microscope findings: the blood cells of *Littorina littorea* differ with regard to the nucleoplasmic ratio, nuclear shape and position, the development of their cell organelles and size of glycogen deposits. According to the hypothesis proposed by Cheng and Cali (Cheng & Cali, 1974) glycogen deposits may appear within phagocytic cells as a result of metabolic conversion of compounds derived from phagocytosed material during intracellular digestion. If this is the case, the size and form of the glycogen deposits should reflect, at least partially, the phagocytic abilities of the cell and, hence, the degree of its maturation. Since the variations in cell structure described apparently represent quantitative rather than qualitative differences, haemocytes of *L. littorea* seem to constitute a pool of cells at different stages of maturation. We attribute periwinkle haemocytes of I, II and III morphotypes respectively to juvenile, intermediate and mature cells.

This suggestion is also supported by the fact that only one morphotype of blood cell incorporates BrdU. BrdU-labelled cells differ from other cells with respect to their morphology (similar to juvenile cells but slightly larger), and may be considered as a specialized subpopulation of blast cells. Hypothetically, relatively few blood stem cells (still unidentified) may produce the haemoblasts which can proceed through a cell cycle several times before differentiation into mature haemocytes which then appear to lose the ability to divide.

The presence of both haemocytes in the DNA synthesis phase and mitotic cells in the haemolymph of *L. littorea*, provides strong evidence that the haemopoietic process in this prosobranch takes place throughout the blood circulating system. Indeed, our parallel histological study (not published) failed to reveal any specialized haemocyte-producing organs or tissues in the periwinkle. A similar way of haemopoiesis is attributed to *Lymnaea stagnalis* by Sminia and colleagues (Sminia et al., 1983). However, other authors reported about specialized 'amoebocyte-producing organ' (APO) located in reno-pericardial region of relative limnaeid *L. truncatula* (Rondelaud & Barthe, 1982) and planorbid *B. glabrata* (Sullivan & Spence, 1994).

Significant individual and temporal variations in the size of the haemoblast subpopulation reflect the highly varying physiological status of individual snails and (or) different levels of defence system activation. Indeed, in the White Sea, the seasonality of biological events is very pronounced due to the rather severe climate. Subzero temperatures suppress the activity of periwinkles and its potential pathogens (bacteria, fungi, trematodes etc.) for more than half of the year, from September until late May. Presumably, at the beginning of the biological summer (June) when temperatures rise above zero a 'pathogen releasing' phase may occur. This pathogen presence may significantly activate the internal defence system of *Littorina littorea* (which is rather relaxed after the prolonged winter anabiosis) and lead to a renewing of the

haemocyte population. Our data on BrdULI monitoring revealed its highest level in June and supported this suggestion.

Monitoring of the periwinkle haemogram has shown minimal haemocyte concentration in haemolymph of *L. littorea* during the breeding period (beginning of July). The first concentration maximum (June) most probably caused by renewing of cell population as mentioned above. However, the second one (early August) could be evoked by physiological necessity of any long-living invertebrate to lyse the gonad tissue after spawning. This seasonal pulsing of *Littorina* gonad volume was reported in several studies (James, 1965; Fretter, 1980). We assume that haemocytes can play a central role in this process. Thus, the second blood cell concentration maximum is hypothetically due to haemocytes coming out from gonad tissues into circulation after histolysis because it was not accompanied by an increase in haemoblast proliferation rate.

Another finding of the haemogram monitoring is a gradual decrease in the percentage of intermediate haemocytes and accumulation of mature ones during the summer season. Most likely the 'ageing' of the haemocyte population by the end of the summer is caused by the necessity to establish a winter store of glycogen. Whether this phenomenon is due to energetic needs or reflects still enigmatic processes of the periwinkle cell biology remains to be determined. In any case, the haemogram of *Littorina littorea* developed in this study gives us a reliable instrument to investigate the status of the internal defence system of the snail under different natural and experimental conditions. The finding of haemoblasts in periwinkle haemolymph could open new opportunities for haemocytic cell line establishment.

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