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Short Communication

Using flow cytometry to detect haemic neoplasia in mussels (*Mytilus trossulus*) from the Pacific Coast of Southern British Columbia, Canada

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ABSTRACT

Flow cytometry was investigated as an alternative to visual haemocytology for potentially higher-throughput and less subjective detection of neoplasia in *Mytilus trossulus*. In contrast to previous studies of ploidy in the *Mytilus* spp. complex, distinct tetra- and pentaploidal neoplastic cells were rare and a wide range of aneuploidy peaks from 1.4n to 5.5n were detected for late-stage leukemic animals. There was no correlation between aneuploidy and the number of diseased cells for early and intermediate disease stages. Formation of aneuploidy and neoplasia progression might not be simultaneous, and DNA content analysis using flow cytometry was only useful for detecting late stages of the disease.

1. Introduction

Bivalves are used in biomonitoring programs due to their widespread geographic distribution and important role in ecosystem health (Gosling, 1992; Viarengo et al., 2007). *Mytilus trossulus* is the species endemic to the Pacific North West Coast of British Columbia, Canada and there is interest in using prevalence of a leukemia-like disease of the haemolymph, referred to as haemic neoplasia (HN), as a sub-lethal endpoint to monitor for environmental effects from municipal and other effluents. Haemic neoplasia was first described for *M. trossulus* from Yaquana Bay (Oregon, USA) and Sequim Bay (WA, USA) (Farley, 1969). The disease was classified as leukemia because of continuously dividing neoplastic cells in the haemolymph, which eventually penetrate various tissues such as connective tissue, gonads, mantle and foot (Barber, 2004). The disease can contribute to mortalities of *M. trossulus* up to 75% over the annual season in the Pacific North West (Bower, 1989). Neoplasias have been reported for many other mollusks, such as clams and cockles (Diaz et al., 2011; Le Grand et al., 2011; Yevich and Barszcz, 1978).

The most commonly used method for detecting HN is examination of haemolymph under a microscope to identify diseased cells based their inability to adhere and their high nucleus to cytoplasm ratios (Bower, 2006; Elston et al., 1992). This method is laborious and can be subjective depending on the training and experience of the person performing the analysis. The use of disease-specific antibodies greatly improves diagnosis (Smolowitz and Reinisch, 1993) but these are difficult to produce for many marine bivalves and the expertise and time required to process many samples this way is also prohibitive to large-scale environmental monitoring programs.

Several studies have shown that HN is associated with abnormal haemocyte ploidy in several bivalve species, including clams (Delaporte et al., 2008), cockles (da Silva et al., 2005) and mussels (Bihari et al., 2003; Elston et al., 1990; Moore et al., 1991) (Gonzalez-Tizon et al., 2000). Two distinct types of HN have been described in *Mytilus* spp. based on their DNA content, namely the pentaploid and tetraploid forms, with greater prevalence of the pentaploid form (Moore et al., 1991). The progressive development of HN was found to be accompanied by the sequential development of cells with these higher ploidy levels (Elston et al., 1990). Based on these results, flow cytometry (FC) analysis of haemocyte DNA content was investigated for diagnosis of HN in *M. trossulus* in the near shore environment of Southern British Columbia, Canada.

2. Methods

2.1. Mussel sample collection

M. trossulus specimens 4–6.5 cm long were collected in April 2010 from the intertidal zones of Hopkins Beach (Sunshine Coast, British Columbia, Canada, 49.428114N-123.478997E) and Dufferin

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Beach (West Vancouver, British Columbia, Canada, 49.372174N-123.285277E). Haemolymph samples were taken for analysis of HN and ploidy from 48 of these mussels per beach immediately after collecting them. Specimens (120 from each beach) were submerged at each of two monitoring sites off shore of the beach locations until October 2010 (480 specimens in total). All mussels were removed from the frames and taken to the laboratory for analysis. In the same week, additional mussel specimens were collected from the two beaches (48 from Hopkins Beach and 41 from Dufferin Beach).

2.2. Haemolymph collection and cell staining

Haemolymph collection and haematocytological analysis were performed as described previously (Vassilenko et al., 2010). Samples were classified into three groups: normal (less than 5% neoplastic-appearing cells), late leukemic (greater than 90% neoplastic cells) or transitional (between 10% and 90% neoplastic-looking cells). Two hundred microliters of haemolymph was fixed in 1300 μL of 100% ethanol at room temperature for 30 min and stored at −20 °C until further analysis. For flow cytometry the method described by Delaporte et al. (2008) was adopted. On the day of analysis ethanol was removed after centrifugation at 448*g* for 10 min and cells were allowed to rehydrate in 1 ml of 0.01 M phosphate buffered saline with 1% NaCl at room temperature for 30 min. After another centrifugation at 448*g* for 10 min supernatant was removed and cells were re-suspended in 500 μL of 0.01M PBS with 1% NaCl. Cells were transferred into flow cytometry tubes by filtering through an 80 μm nylon mesh to remove all large particles. Five microliters of DNAse-Free RNase A (catalog number 4817-60-04, FlowTACS™ kit, Trevigen, 20 mg/mL) and 10 μL propidium iodide (1 mg/mL) were added before incubation for 30 min in dark at room temperature.

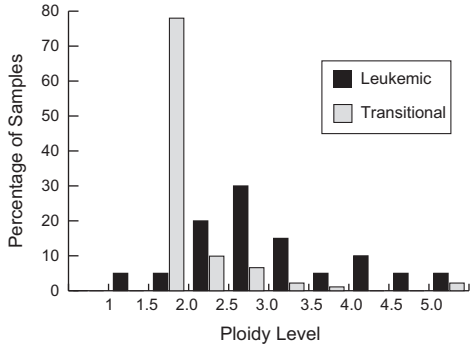

Fig. 2. Histogram plot of the distribution of G0/G1 phase ploidy levels within each of the diseased specimen groups: transitional (5–90% neoplastic cells) and late leukemic (greater than 90% neoplastic cells). The vertical bars represent the percentage of specimens with ploidy in between the values on the *x* axis.

2.3. Flow cytometry data analysis

Samples were analyzed on a fluorescence-activated cell sorter (BD Biosciences FACSCalibur™) instrument in both logarithmic and linear scale modes. For each run, 10,000 events were counted. For most samples run using the linear scale setting, many events were not captured on a plot of fluorescence signal area (FLH-A) versus fluorescence signal width (FLH-W) plot and the method described in (da Silva et al., 2005) for removing doublets could not be used with the logarithmic scale data. Alternatively, plots of side scatter (SSC) versus forward scatter (FSC) were used to select cell groups for ploidy analysis as described in (Allam et al., 2002) and this method proved to be compatible with that of da Silva et al. (2005) for those samples for which both linear and log scale data

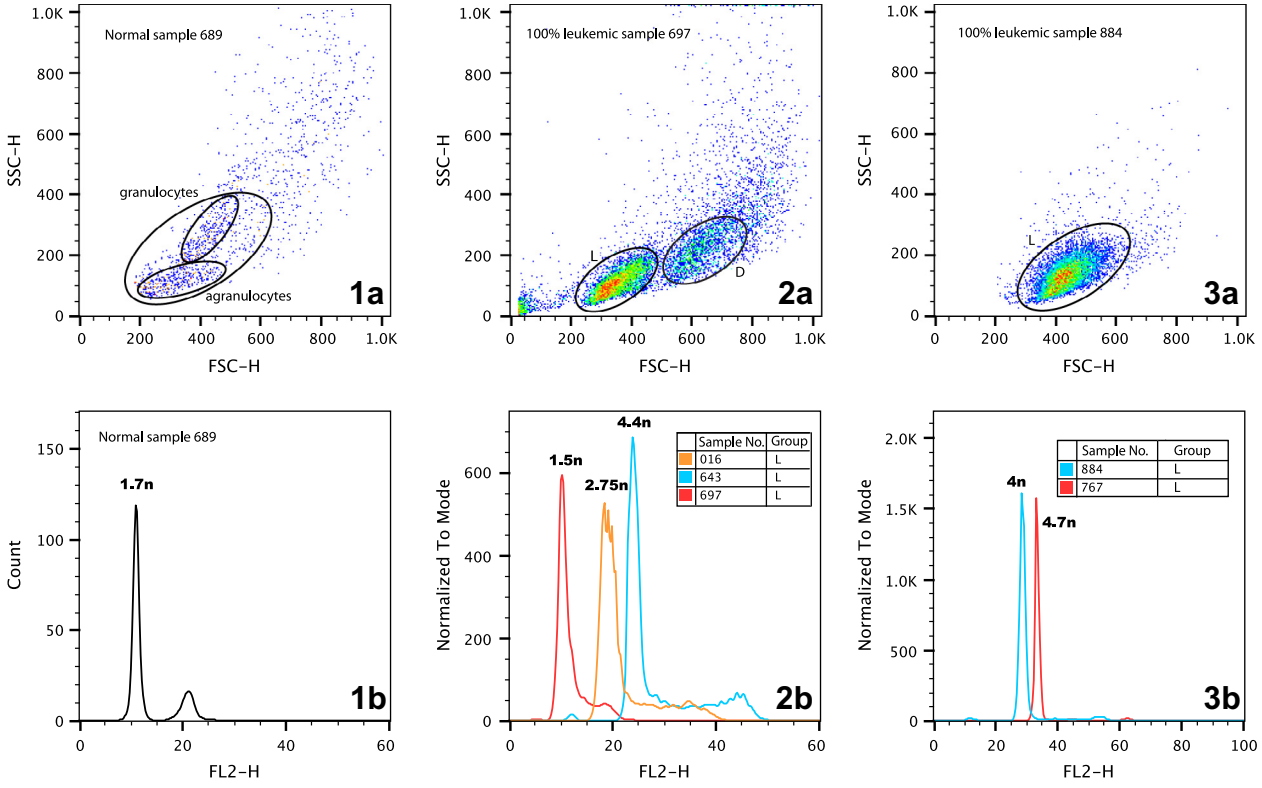

Fig. 1. Side scatter (SSC-H) versus forward scatter (FSC-H) plots depicting cell groups and FL2-H histograms revealing ploidy levels for a typical normal (less than 5% neoplastic-appearing cells) (panels 1a and 1b) and five late stage leukemic (100% neoplastic) (panels 2a, 3a, 2b and 3b) haemolymph samples. Ploidy levels written above or next to the main G0/G1 phase peaks on the FL2-H histograms were determined by comparing the DNA content with that for a sperm sample.