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Aquaculture 292 (2009) 180–188

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Differing cell population structure reflects differing activity of Percoll-separated pronephros and peritoneal leucocytes from barramundi (*Lates calcarifer*)

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ARTICLE INFO

Article history:
Received 13 February 2009
Received in revised form 19 April 2009
Accepted 20 April 2009

Keywords:
Barramundi
Macrophage
Respiratory burst
Phagocytosis
Flow cytometry
Chemiluminescence

ABSTRACT

Aquaculture of barramundi or Asian sea bass (*Lates calcarifer* L) is expanding throughout the Asia-Pacific region in both marine and freshwater systems. Incidence of bacterial and viral diseases is high in this species throughout the region yet little is known about the immune system of this highly adaptable euryhaline fish. Ultimately, most pathogens are eradicated by the phagocytic cells, however there is great diversity and plasticity amongst these cell populations in mammals and in fish. To better understand disease processes in barramundi, Percoll-purified leucocyte populations from haematopoietic tissues of the head kidney were compared with populations isolated from the peritoneal cavity morphologically, cytochemically and in terms of the ability to respond to stimulation, using flow cytometry, light microscopy and fluorimetric/luminometric assays. The peritoneal cells comprised predominantly of macrophages and putative mature monocytes whilst the head kidney cells comprised lymphocytes, including immunoglobulin-positive B-lymphocytes, some small monocytes and macrophages. The differing population structures were reflected in the ability of the cells to respond to stimulation with either lipopolysaccharide or phorbol myristate acetate, as the chemiluminescence response of peritoneal cells was 7 to 9-fold higher than head kidney cells. Both populations were capable of being primed by LPS, but the kinetics differed, with optimal priming in peritoneal cells occurring after 6 h exposure whilst head kidney cells required at least 24 h exposure to LPS for optimal priming. Both head kidney and peritoneal populations produced nitric oxide in response to stimulation with LPS and interferon gamma, but again, response was higher in peritoneal cells. The implications of the differing population structures and activities amongst these cells should be considered when developing models for further study of host–pathogen interactions in this increasingly important fish.

1. Introduction

Barramundi (*Lates calcarifer*) is an iconic fish with high cultural significance and economic value in Australia. Commonly known as Asian sea bass, barramundi are widely farmed throughout the Asia Pacific region and in Australia barramundi represent the second largest finfish aquaculture sector after Atlantic salmon (Love and Langenkamp, 2003). The barramundi industry has shown strong growth in Australia lately with a 21% increase in production in 2003/2004 and more than 20% increase over 2005/2006 (Lobegeiger and Wingfield, 2007). Farmed barramundi are prone to a number of infectious diseases including bacterial infections by *Aeromonas* sp, *Flexibacter columnaris*, *Vibrio harveyi* and *Streptococcus iniae*. Nodavirus represents the most prevalent viral disease of farmed barramundi (Munday et al., 1994; Bromage et al., 1999; Bromage and Owens, 2002; Agnew and Barnes, 2007). In spite of the industry's value and the restrictions imposed upon its growth by disease, little is known about the barramundi immune system. Research to date has focused predominantly on the adaptive immune system of barramundi, with immunoglobulins having been purified and characterised on several occasions (Bryant et al., 1999; Crosbie and Nowak, 2002; Bromage et al., 2004), and both serum and mucosal antibody responses to killed vaccines determined in freshwater and seawater (Delamare-Debouteville et al., 2006). However, the innate immune system of barramundi remains uncharacterised.

The innate immune response of fish is the first line of defence against invading pathogens and comprises a variety of immune defence mechanisms involving both cellular and humoral components. The cellular defences include a diverse population of cells that act both independently and collaboratively and fulfill numerous roles in both adaptive and innate immunity, including pathogen detection, signalling, phagocytosis and destruction. Ultimately, most invading

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doi:10.1016/j.aquaculture.2009.04.030

bacterial species are eradicated by phagocytic cells, including the macrophages and neutrophils (Ellis, 1977b). Fish phagocytic cells undergo a series of responses to specific stimuli and enter a state of activation resulting in increased microbicidal capability. Activated phagocytic cells such as macrophages and neutrophils also have the potential to manifest an increase in respiratory burst activity, a process where the electron of molecular oxygen is reduced into highly reactive superoxide anion (O_2^-) by a membrane enzyme, NADPH oxidase which is generated from glucose via the hexose monophosphate shunt (Babior, 2000). In unstimulated phagocytic cells like neutrophils, this enzyme complex is inactive and only assembled upon cell activation by a variety of factors including opsonized bacteria or zymosan, aggregated IgG, and protein kinase C activators such as phorbol myristate acetate (PMA) (Meseguer et al., 1994; Tahir and Secombes, 1995; Babior, 2000; Sarmento et al., 2004). Superoxide anion serves as the precursor for the formation of other ROS (Hampton and Winterbourn, 1999). Hydrogen peroxide (H_2O_2) is rapidly formed from superoxide anion by spontaneous dismutation or enzymatic dismutation by superoxide dismutase (100). In the presence of transition metal such as free iron (Fe^{2+}), the formation of hydroxyl radicals ($\cdot OH^-$) can be spontaneously degenerated from superoxide anion and hydrogen peroxide via iron-mediated Haber–Weiss type Fenton reactions (Bullen, 1987). The hydroxyl radicals are more reactive than peroxide and may interact with nitric oxide (NO) to form peroxynitrite. In addition, superoxide anion and hydrogen peroxide are used as precursors for the production of the bactericidal oxidants, the oxidized halogens group (e.g. ClO^-) and the oxidizing radicals group (e.g. $\cdot OH^-$).

The quantity of ROS produced, therefore, has been used as an indicator of the intensity of the innate immune response and the health status of the organism. Indeed, respiratory burst activity of phagocytes in response to various stimuli such as infection, environmental contaminants, growth factors and other immunomodulators has been extensively used as a reliable measurement of the immune response of a variety of fish species.

In higher vertebrates haematopoietic stem cells from which many of these leucocyte populations are derived originate in the bone marrow (Cooper, 2003; Ellis, 1982). Teleosts do not have bone marrow, but haematopoietic tissues are evident in the head kidney, spleen and liver (Ellis, 1977b). Haematopoiesis is first seen in the head kidney early in ontogeny (Ellis, 1977a) and it is believed that the low-pressure renal portal system coupled with environmental radiation shielding resulting from the large amounts of melanin in the head kidney provide an ideal microenvironment for progenitor stem cells of the leucocyte population (Randall, 1970; Cooper et al., 1980).

Progenitor cells originating in the haematopoietic tissues give rise to a number of specific functional lineages via the myeloid lineage precursor including the granulocytic lineage which includes neutrophils, and the monocytic/macrophage lineage. These professional phagocytic cells are of great interest in the study of health and immunity as their differing antimicrobial activities and ability to mobilise into infected territories has been clearly established in a number of fish species (Afonso et al., 1998; do Vale et al., 2003).

Inflammatory response in fish is considered to be biphasic, with an initial recruitment of neutrophils to the site of infection followed by the arrival of monocytes and macrophages (Reite and Evensen, 2006). Thus leucocyte populations isolated from haematopoietic tissues may be expected to differ in population structure and in their interactive function from those isolated from sites of infection or inflammation.

These diverse leucocyte populations have been described in a number of finfish species including rainbow trout (Afonso et al., 1997), Atlantic salmon (Jorgensen et al., 1993), European sea bass (Do Vale et al., 2002), goldfish (Belosevic et al., 2006), Atlantic cod (Sorensen et al., 1997), gulf killifish (Haszell and Rice, 1998) and spotted wolfish (Norum et al., 2005). To date little is known of the population structure and function of the leucocytes of barramundi.

The aim of the present study was, therefore, to characterise leucocytes from the head kidney (representative of haematopoietic tissue) and from casein-stimulated peritoneal cavity as a model site of inflammation. Populations were investigated structurally by light microscopy, flow cytometry and imaging flow cytometry. The activation kinetics of these populations in response to various stimuli was also determined by measuring luminol-enhanced chemiluminescent response. As head kidney macrophages are often chosen as models for assessing immunocompetence in fish (Cook et al., 2003; Bagni et al., 2005), or resistance of fish pathogenic bacteria to phagocytic attack (Sharp and Secombes, 1993; Barnes et al., 1999, 2002; do Vale et al., 2003; Zlotkin et al., 2003), an objective assessment of the structure and capabilities of head kidney leucocytes (HKL), compared to those arriving at a site of inflammation should provide further insight into their suitability as a representative models for the study of host–pathogen interactions.

2. Materials and methods

2.1. Experimental animals and maintenance

Barramundi, *L. calcarifer*, approximate weight 100 ± 20 g were purchased from a commercial farm (Barramundi Australia, Stapylton, Australia) and maintained in recirculating filtrated water at a temperature of 25–28 °C and salinity of 10 ppt, in 500 L round plastic tanks. Water quality was maintained at pH 6.5–7.5, ammonia <2 ppm, nitrite <5 ppm and nitrate <40 ppm with mechanical and biological filtration, and periodic water changes as required. Before the experiment, the fish were acclimated for 2 weeks, and during this period, they were fed twice daily to apparent satiation with a commercial diet (Marine Float 4 mm, Ridley Aqua Feeds, Narangba, Australia).

2.2. Leucocyte isolation and cell culture

Macrophages from head kidney and peritoneal cavity were harvested, purified and maintained as described previously (Secombes, 1990). For stimulation of the peritoneum, fish were anaesthetised with Aqui-S (Aquatic Diagnostic Services, Wilston, Australia) in accordance with the manufacturer's instructions, and then injected with 1 mL of 12% casein (sterile, in phosphate buffered saline, PBS) into their peritoneal cavity 24 h before collection of macrophages. Prior to the isolation of peritoneal macrophages, fish were euthanised with overdose Aqui-S and then exsanguinated by cutting the ventral aorta. An aliquot (5 mL) L-15 medium containing 2% Foetal Bovine Serum (FBS, Invitrogen, Melbourne, Australia) penicillin/streptomycin (P/S) (Invitrogen, Melbourne, Australia), and 10 U mL^{-1} heparin (Sigma, Castle Hill, Australia) was injected asceptically into the peritoneal cavity using a syringe fitted with a 25 G needle. The body cavity was then massaged for 30 s to disperse the medium and the lavage containing leucocytes was withdrawn using syringe fitted with a 19 G needle very carefully to prevent bleeding. Collection of head kidney macrophages was performed simultaneously with harvest of the peritoneal macrophages. The pronephros was dissected out aseptically and pushed through a 100 mm nylon mesh in L-15 medium containing 2% FBS, 1% P/S, and 10 U mL^{-1} heparin. The suspensions of head kidney and peritoneal cells were then layered onto a discontinuous (34%/51%) Percoll density gradient and centrifuged at 450 ×g for 25 min at 4 °C. The band lying at the interface was collected and washed twice with L-15 medium containing 1% FBS and 1% penicillin/streptomycin (P/S). Concentration of viable cells was determined by Trypan blue exclusion. Cells (100 μL) were seeded in 96 well tissue culture treated microtitre plates (Greiner, Germany) at a concentration of 10^7 cells mL^{-1} in L-15 medium with 1% FBS and 1% P/S. Both cell populations were allowed to adhere for 2 h at 28 °C and then washed twice with L-15 medium to remove the unattached cells. The adhered cells were maintained in L-15 with 1% FBS and 1% P/S at 28 °C.

2.3. Analysis and sorting by flow cytometry

Head kidney and peritoneal exudate cells were analysed within 1 h of isolation. Flow cytometric analysis was performed with a FACScalibur flow cytometer (BD Biosciences) and data were analysed using the Lysis II software package (BD Biosciences). A minimum of 10,000 gated leucocytes were analysed per tube with the forward scatter (FSC) threshold set at 645 whilst the side scatter threshold was set at 220. Cell populations were sorted using the BD FACSaria cell sorter (BD Biosciences) to collect gated populations based on differing FSC and SSC characteristics. Cell suspensions initially comprised of 10^7 cells mL^{-1} and were sorted at a rate of 10^4 cells s^{-1}. The sorted cells were collected and then analysed by light microscopy of cytospin preparations stained for morphological and cytochemical analysis as described in Section 2.4 below.

Putative B-lymphocytes were differentiated by flow cytometry on the basis of presence of surface immunoglobulin. Briefly, cells were washed once in PBS + 3% FBS, harvested by centrifugation at 1250 ×g at 4 °C for 5 min then incubated on ice for 60 min with a monoclonal mouse *vs* barramundi IgM (20 μg mL^{-1}) (AquaMab F02, Aquatic Diagnostics Ltd., Stirling). Cells were washed twice in PBS + 3% FBS prior to labelling for 30 min with goat *vs* mouse phycoerythrin conjugated polyclonal antibody (P9287, Sigma, Castle Hill, Australia) diluted 1:100. Cells were analysed and counted with an AMNIS ImageStream 100 flow cytometer (Amnis Corporation, Seattle, Wa). Five thousand events were recorded per sample and the data analysed using IDEAS software (Amnis Corporation, Seattle, Wa).

2.4. Morphological and cytochemical identification by light microscopy

Differential cell counts of the cell suspensions were performed on cytospin preparations. After Percoll separation, the cells were washed once and resuspended in L-15 supplemented with 5% FBS and 1% P/S. Fifty μL of the final cell suspension was cytocentrifuged (Shandon Cytospin 4 Cytocentrifuge, Thermo Scientific, Sydney, Australia) at 60 ×g for 5 min. The cells were then air dried, and stained with Diff Quick (Sigma, Castle Hill, Australia) according to the manufacturer's instructions. Cells were identified under light microscopy and 5 fields containing approximately 100 cells per field were counted to determine the proportion of each cell type within the population.

A commercial leucocyte peroxidase staining kit (Sigma, Castle Hill, Australia) was used for the histochemical demonstration of peroxidase enzymatic activity in the mixed leucocyte populations to facilitate the differential counting between macrophages, neutrophils and lymphocytes. Peroxidase staining was carried out on cytospins in accordance with the manufacturer's protocol.

Detection of specific and non-specific leucocyte esterase was performed using a commercial Naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase (Sigma, Castle Hill, Australia). Cytospins of HK and PT leucocytes (30,000 cells $cytospin^{-1}$) were processed through the double staining esterase procedure as per the manufacturer's protocol (Sigma, procedure no. 91).

2.5. Respiratory burst activity by luminol-enhanced chemiluminescence

The respiratory burst activity of the head kidney cells following stimulation with a number of immunostimulants was determined using protocols luminol-enhanced chemiluminescence as described previously (Nikoskelainen et al., 2005) with minor modification. In order to examine the effect of different immunomodulators, triplicate wells of macrophages (10^6 cells $well^{-1}$) were incubated with phorbol myristate acetate (PMA) at concentrations of 10, 50 and 100 ng mL^{-1}, and LPS or peptidoglycan (PTG) at 10, 50 and 100 mg mL^{-1}. To each well, 10 μL of 10 mM luminol in 0.2 M borate buffer pH 9.0, 280 ml HBSS pH 7.4 and the immunostimulant were added to a final volume of 300 ml. The chemiluminescence (CL) emissions of the phagocytes were measured with a Fluostar Optima plate reader (BMG Labtech, Melbourne, Australia) every 3 min for 3 h at 27 °C.

2.6. Determination of peroxide production by flow cytometry using dihydrorhodamine 123 (DHR123)

Intracellular hydrogen peroxide production was measured in leucocyte populations prior to and 15, 45 and 60 min following triggering with PMA (100 ng mL^{-1}) flow cytometrically using a method described previously (Ortuno et al., 2000; Joerink et al., 2006). Briefly, DHR123 (Sigma, Castle Hill, Australia) was added to the cells in culture at 10 μg mL^{-1} and the cells then stimulated with 100 ng mL^{-1} PMA. After incubation for the designated time, cells were detached from the culture plates with trypsin EDTA (Invitrogen, Melbourne, Australia) then transferred to flow cytometer tubes for analysis. To determine the effect of culture and subsequent detachment on cell viability, replicates of each sample were incubated with propidium iodide (0.1 μg mL^{-1}) (PI, Fluka from Sigma, Castle Hill, Australia). Forward scatter (FSC) and side scatter (SSC) characteristics of 10,000 events per sample were acquired in linear mode and fluorescence intensities at 530 nm for DHR123 and 610 nm for PI were acquired in logarithmic mode using a BD LSRII Analyser flow cytometer.

2.7. Assay of nitric oxide production by leucocyte populations

Production of nitric oxide in the two cell populations was determined using a commercial microplate-based fluorometric detection system (Sigma, Castle Hill, Australia) based on the NO-specific fluorochrome, DAF-DA. Cells were cultured for 48 h at 28 °C in black 96-well flat-bottomed microtitre plates. A preliminary dose response experiment was performed in which cells were stimulated for 7 h with 0.1 μg mL^{-1}, 10 μg mL^{-1} of LPS and 0.01 μg mL^{-1}, 1 μg mL^{-1} interferon γ (IFN-γ) (mouse recombinant expressed in *E. coli*, Sigma, Castle Hill, Australia), independently and in combination. Unstimulated cells were used as controls and a cell-free reagent-only blank was employed. To confirm the specificity of the detected response, a specific inducible nitric oxide synthase (iNOS) inhibitor, diphenyleneiodonium chloride (DPI) (Sigma, Castle Hill, Australia, supplied with the detection kit) was added to replicate wells of each reaction at a final concentration of 8 μM. After 7 h, the reagents were removed and the fluorochrome DAF-DA was added, incubated and read in accordance with the manufacturer's instructions with a BMG Fluostar Optima Fluorometer (BMG Labtech, Melbourne) using a 485 nm excitation filter and an emission filter of 520 nm.

Following establishment of the optimum dose, a time course experiment was conducted using peritoneal and head kidney cells cultured for 48 h. To minimise excessive handling of the cells, a single microplate was used for each experiment. All wells were seeded simultaneously with peritoneal and head kidney cells and cultured for 48 h at 28 °C. Stimulants (a mixture of 10 μg mL^{-1} LPS and 1 μg mL^{-1} IFN-γ) were added to triplicate wells of each cell type 12 h, 6 h and 3 h prior to reading the plate. Controls and reagent blanks were prepared as described above. Twelve hours following addition of the first stimulants, the supernatants were removed from all wells and assayed for free nitrate with DAF-DA as described above.

2.8. Statistical analysis

Data were analysed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, Ca). Cell counts were analysed by one way analysis of variance (ANOVA) followed by Students *t*-test. Results are expressed as means ± SD. Luminol chemiluminescence data were smoothed using weighted average of the nearest 5 neighbours (Nikoskelainen et al., 2006). Total luminescence was determined by integrating the smoothed curves to determine the area under the curve. Integrated data for independent

replicate tests were then analysed by one-way ANOVA followed by Tukey's multiple pairwise comparison post-tests.

3. Results

3.1. Leucocyte populations from head kidney and peritoneum are different

Fig. 1A shows a typical FACS analysis of PTL. There were three distinct sub-populations termed P2, P3 and P4 based on forward scatter (FSC) and side scatter (SSC) as discriminating parameters. The sorted sub-populations were prepared by cytospin and stained with Diff Quick stain for morphological characterisation and putative identification. The population P2 was dominated by small monocytes (Fig. 1A/P2). The P3 fractions contained primarily larger cells including macrophages and small percentage of small monocytes, large lymphocytes and neutrophils (Fig. 1A, Table 1). The P4 fraction consisted of granular cells such as neutrophils and eosinophils indicated by high SSC values (Fig. 1A, Table 1). HKL sub-populations were also separated into three fractions called P2, P4 and P5 (Fig. 1B). The P2 population comprised small cells about the size of lymphocytes as confirmed by morphological studies. A sub-population that had the largest cell size found in HKL fell into region P4 that contained large lymphocytes, small monocytes and a small percentage of neutrophils. The P5 fraction was comprised of granulocytes based on its higher granularity and medium cell size. Data obtained by light microscopy were supported by results obtained by imaging flow cytometry that indicate a diverse population of smaller cells from head kidney compared with a homogeneous population of larger macrophage-like cells from peritoneal exudate. To try to elucidate the nature of the smaller cells in HKL, a monoclonal antibody directed against barramundi IgM was used to label putative B-lymphocytes in both populations followed by subsequent analysis by imaging flow cytometry. Results indicated that the HKL contained a significant proportion of B-lymphocytes, with more than 50% of the viable cells staining for surface IgM (Table 1). In contrast, very few cells were stained with the anti-barramundi IgM antibody in peritoneal exudate (4.06%).

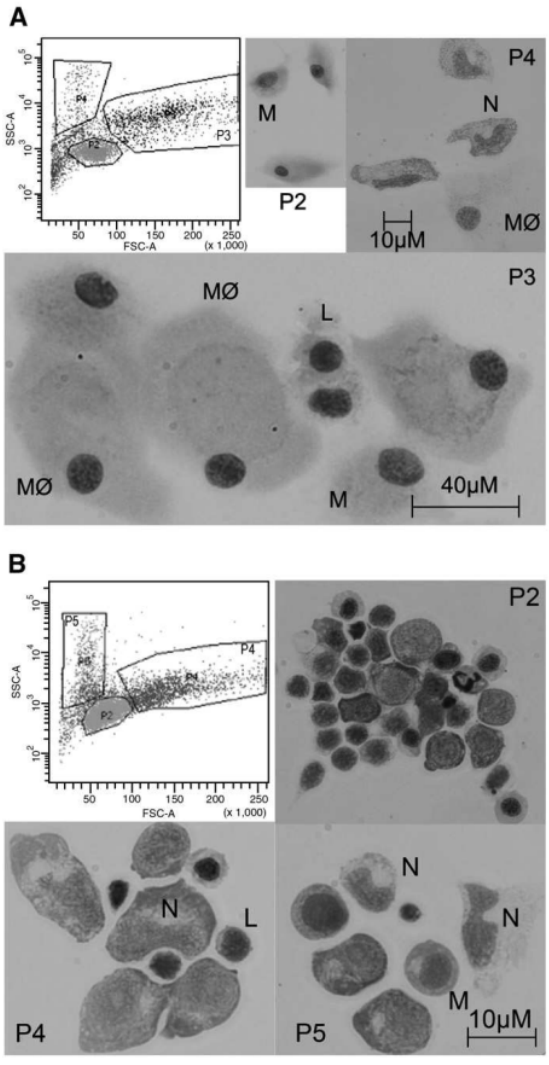

Fig. 1. Flow cytometric analysis of leucocyte populations. Leucocytes from head kidney (A) and peritoneum (B) were analysed by flow cytometry. Gated populations were separated and cytospin preparations analysed by light microscopy (Magnification ×1000). In each case, MØ = macrophages; M = monocytes; L = lymphocytes; N = neutrophils.

Table 1
Leucocyte population structures.

Cell type	Head kidney	Peritoneal
Total Lymphocytes (B-lymphocytes, IgM+) and progenitor cells	64.3 (51.08)	38.2 (4.08)
Neutrophils and small monocytes	7.6	4.6
Macrophages, larger monocytes and granulocytes	16.9	33.8

Leucocyte populations were analysed by flow cytometry and AMNIS imaging flow cytometry. Proportions of each gated population were determined as a percentage of viable cells in each population. Putative cell type was assigned by morphological characteristics in Diff Quick-stained cytospin preparations of each gated population under light microscopy. B-lymphocytes were identified by flow cytometry as positive for IgM using a monoclonal antibody.

3.2. Activation of head kidney and peritoneal leucocyte populations

To determine the physiological relevance of the differences in cell populations, a series of experiments were conducted to investigate the activation of the cells by differing stimulants, including LPS, PTG and the surface receptor independent protein kinase C activator, PMA. Activities were determined by luminol-enhanced chemiluminescence and data are presented in Fig. 2, as total luminescence generated over the 3 h period of the assay. In terms of total luminescence, peritoneal cells produced significantly more ROI in response to stimulation by PMA regardless of the concentration used (Fig. 2). LPS also stimulated respiratory burst, but only when either 50 μg mL^{-1} or 100 μg mL^{-1} were used. Respiratory burst was not activated in peritoneal cells by PTG at any concentrations used, or by LPS at 10 μg mL^{-1} (Fig. 2A).

In contrast the head kidney cells were only activated significantly higher than controls by PMA (Fig. 2B). Whilst there was an increase in chemiluminescence with 10 and 50 μg mL^{-1} LPS, this was not significantly different from non-stimulated controls (Fig. 2B). In general the total ROI produced by HKL was much lower than that detected in PTL (Fig. 2A, B).

As the total ROI produced by unstimulated PTL was generally higher than that detected in HKL, data were also analysed as relative luminescence, normalising the luminescence detected in stimulated cells against that detected in controls. When data were analysed in this way, the fold increase in ROI produced by HKL (~7 to 8-fold) in response to PMA was very much higher than the increase in ROI produced by PTL (~3-fold) with respect to unstimulated controls,

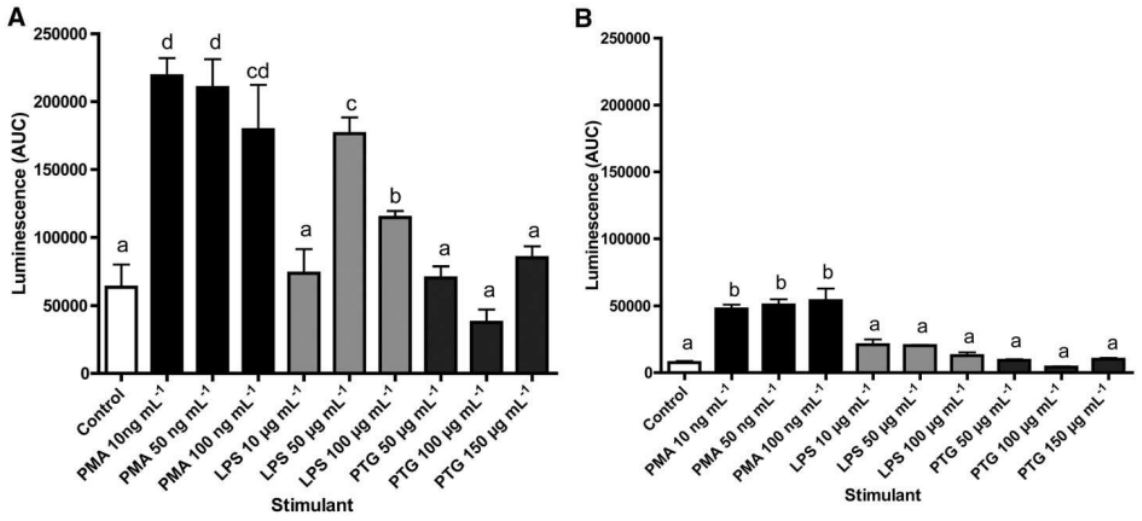

Fig. 2. Direct stimulation of respiratory burst in peritoneal (A) and head kidney (B) leucocyte populations assayed by chemiluminescence. Percoll-separated HKL or PTL (1×10^6 mL^{-1}) were incubated in medium containing 10 mM luminol in 0.2 M borate buffer pH 9.0, 280 mL HBSS pH 7.4. At time zero the stimuli were added and chemiluminescence (light units) was measured at 3 min intervals over a period of 3 h at 27 °C. Data are means ± SEM of replicate assays with leucocytes from 5 fish.

whilst the fold change produced in response to LPS was similar in both populations.

3.3. HKL and PTL can be primed by LPS

To determine whether cells could be primed by LPS prior to stimulation, populations cultured overnight were exposed to the two lowest LPS concentrations used in the stimulation experiments for 3, 6 and 24 h, prior to triggering with PMA. Priming was seen in both cell populations in response to LPS at both concentrations employed, as indicated by increase in ROI induced luminescence (Fig. 3). There was both a time and treatment effect, however the priming effect was different for PTL compared to HKL. When PTL were primed, significant priming was detected after 3 h, with peak priming effect seen after 6 h, and further exposure (up to 24 h) resulting in reduced PMA-induced ROI production (Fig. 3A). In contrast, no significant priming was detected after 3 h in HKL, but increasing respiratory burst activity in response to PMA was detected with increased priming time up to 24 h (Fig. 3B).

3.4. Hydrogen peroxide production by PTL and HKL by flow cytometry

As chemiluminescence measures total ROI production, peroxide production was specifically determined using the fluorochrome dihydrorhodamine (DHR123) by flow cytometry. Following stimulation with PMA, both HKL and PTL populations showed an increase in

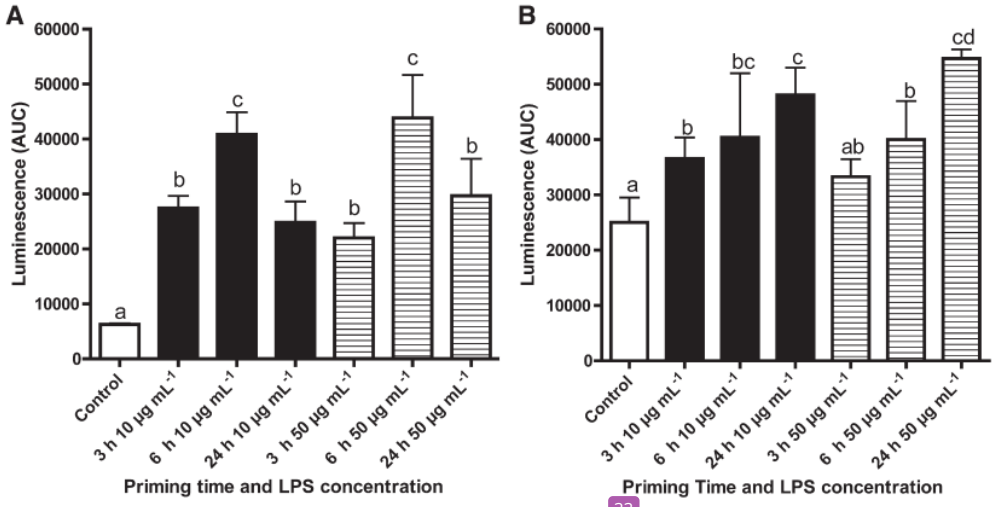

Fig. 3. Priming effect of LPS on peritoneal exudate and head kidney cells. Peritoneal exudate or head kidney cells were incubated with 10 µg mL^{-1} or 50 µg mL^{-1} LPS for 3, 6 or 24 h prior to triggering with PMA 0.01 µg mL^{-1} (A) PTL (B) HKL.

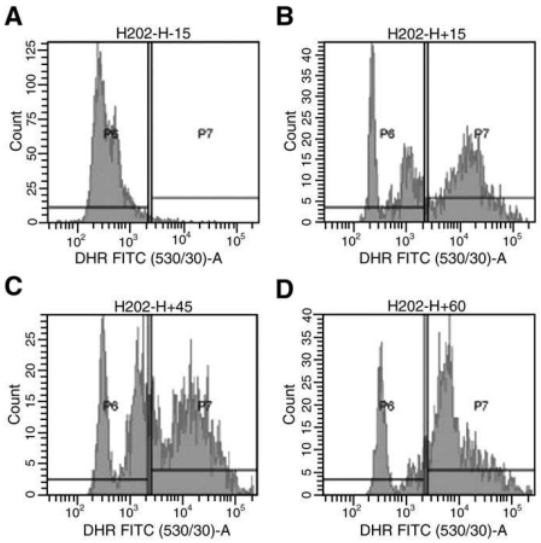

Fig. 4. Flow cytometric determination of hydrogen peroxide production by head kidney leucocyte populations. Histograms plotting cell count against fluorescence of head kidney cells in resting state (A) or 15 min (B), 45 min (C) or 60 min (D) following stimulation with 0.1 μg mL^{-1} PMA. Increased fluorescence resulting from hydrogen peroxide production is indicated by a shift to the right on the *x*-axis.

peroxide production over time (Figs. 4 and 5). In common with results determined using chemiluminescence, peroxide production in PTL peaked earlier (45 min) than in HKL which indicated higher peroxide production 60 min following stimulation (Figs. 4 and 5). Disparity between the two populations was also evident in the number of peaks of fluorescing cells recorded from each population: In HKL a large peak

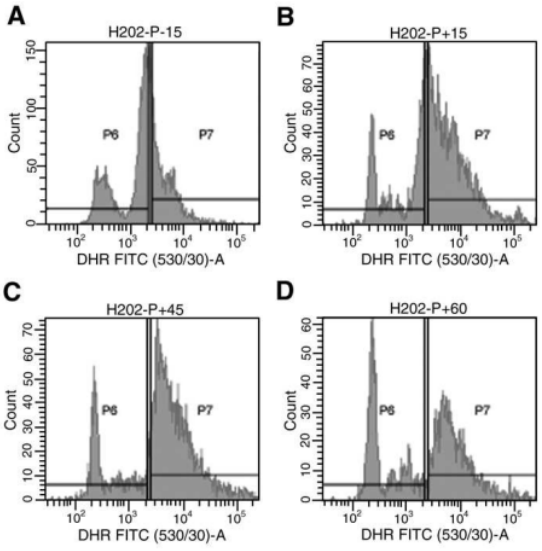

Fig. 5. Flow cytometric determination of hydrogen peroxide production by peritoneal leucocyte populations. Histograms plotting cell count against fluorescence of peritoneal cells in resting state (A) or 15 min (B), 45 min (C) or 60 min (D) following stimulation with 0.1 μg mL^{-1} PMA. Increased fluorescence resulting from hydrogen peroxide production is indicated by a shift to the right on the *x*-axis.

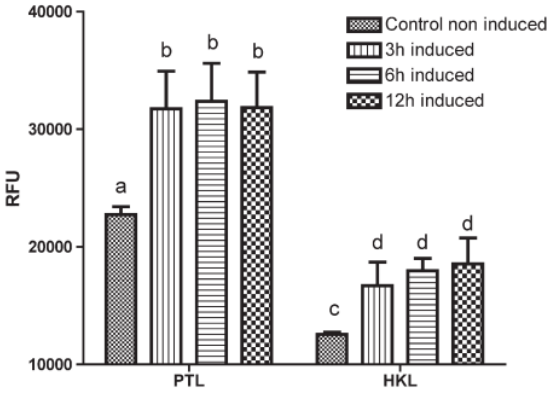

Fig. 6. Nitric oxide production by head kidney and peritoneal leucocyte populations determined by fluorimetry. Inducible nitric oxide synthase (iNOS) was induced by stimulating cell populations with 10 μg mL^{-1} LPS + 1 μg mL^{-1} IFN-g for 3, 6 and 12 h then assaying NO production using the specific fluorochrome, DAF-DA. Results are presented as means ± SEM ($n = 3$) and different letters indicate significant differences between values ($p < 0.01$).

with very low basal fluorescence prior to stimulation (Fig. 4A) separated into 3 peaks, one with basal fluorescence, and two with elevated fluorescence (Fig. 4B, C, D), suggesting response of multiple cell types. In contrast, PTL cells prior to triggering with PMA comprised two peaks, one with similar basal fluoresce to the unstimulated peak recorded in HKL, whilst a second peak, comprising the majority of cells, exhibited a higher basal fluorescence (Fig. 5A). After triggering with PMA, the second peak increased in fluorescence (Fig. 5B, C), with the majority of cells exhibiting high fluorescence after 45 min (Fig. 5C). After 1 h, this peak decreased in size, whilst the peak indicating cells at basal fluorescence increased, indicating cells returning to a resting state, or becoming exhausted (Fig. 5D).

3.5. Peritoneal cells produce more nitric oxide than head kidney cells

To further investigate the nature of the ROIs produced by the HKL and PTL populations, nitric oxide production was assayed in microtitre plates using the fluorochrome DAF-DA. Whilst PTL produced more nitric oxide than HKL, the increase in NO production compared to unstimulated controls by HKL was higher than PTL (Fig. 6). Following induction, rate of production of NO was relatively constant over 12 h in PTL, whereas in HKL there was rapid induction of NO production (3 h), which decreased over the following 9 h (Fig. 6).

4. Discussion

Barramundi head kidney and peritoneal exudate derived leucocytes purified on Percoll gradients comprised of lymphocytes, neutrophils, monocytes, macrophages and other granulocytes. The peritoneal cells were larger in size and consisted of more homogenous monocyte/macrophage-like cells. The head kidney cell population, on the other hand, comprised predominantly of smaller cells that appeared to be granulocytes, neutrophils, monocytes and particularly lymphocytes. Imaging flow cytometry analysis supported the light microscopy indicating substantial differences in the structure of these cell populations. Three clusters of cells were observed from HKL and PTL of barramundi after separation on 34%/51% Percoll density gradients. Cells with small FSC/SSC profiles (P2 in PTL and HKL) mainly consisted of small lymphocytes. A cell population with increased SSC characteristics (P4 in PTL, P5 in HKL) consisted of granular cells such as neutrophils and some eosinophils. Cell populations with low SSC but high FSC values (P3 in PTL, P4 in HKL) consisted mainly of large monocytes, macrophages

and a small percentage of large lymphocytes. Similarly, van Kemenade et al. (1994) reported three distinct leucocyte populations in carp pronephros examined by flow cytometry based on FSC/SSC profiles (van Kemenade et al., 1994). Three sub-populations were also obtained from the head kidney of dab, *Limanda limanda* (Skouras et al., 2003) and of Atlantic cod, *Gadus morhua* (Ronneseth et al., 2007) with similar cell profiles to those identified in the current study. However, only two populations were found in leucocytes of sea bass isolated from peripheral blood following separation on 51% Percoll density gradients (Esteban et al., 2000) and in carp peripheral blood leucocytes (Nakayasu et al., 1998). Clusters of cells with low FSC/SSC values consisted of erythrocytes, thrombocytes and lymphocytes (Esteban et al., 2000), whilst a second cluster, characterised by large cell size, contained granulocytes, monocytes and macrophages (Esteban et al., 2000). In the context of previous research and the results presented here, it is evident that the differences in the number of fractions or sub-populations and the morphology of the cells in each cluster are dependent on the leucocyte sources, the method used and the physiological status of the fish or a combination of these variables.

Head kidney, as a haematopoietic tissue where progenitor cells originate, predominately contains immature, undifferentiated cells which will be recruited to and mature in the tissues or the infectious sites (Ellis, 1977b). The peritoneally-derived leucocytes on the other hand, were harvested following an injection of casein and, as expected, these cells were more mature, perhaps resembling the sort of populations of cells that would be mobilised to deal with an infection event. In sea bass, unstimulated peritoneal exudate comprised 97% lymphocytes and macrophages and a small percentage of neutrophils (Afonso et al., 1997; Ellis, 2001), and in trout consisted entirely of macrophages (Zelikoff et al., 1991). These findings suggest that even in the resting state, the peritoneal population contains mainly large mature cells. The proportion of leucocytes in fish can alter drastically under stress or infection (Ellis, 2001). Stress related to temperature changes resulted in significant changes in haematology and blood biochemistry, and in particular the number of leucocytes of the tench, *Tinca tinca* (de Pedro et al., 2005), and of Atlantic salmon (Pettersen et al., 2005). Studies in carp and rainbow trout with a haemoflagellate infection indicated a depletion of leucocyte numbers in head kidney due to an influx of granulocytes into the peripheral blood (Scharsack et al., 2003).

We found that analysis of barramundi leucocytes by flow cytometry has certain advantages over other methods as the total leucocyte population could be analysed without selectivity or excessive handling and the samples could be sorted and recovered if required. In this study however, it was difficult to recover the sorted cells for functional assays and differential counting due to the "sticky" nature of the cells. Although we were able to collect more than 10^5 cells in each 5 mL collection tube, only low numbers of cells could be recovered on the cytospin slides. Several methods have been tried to reduce adherence and aid cell recovery, such as increasing the FBS concentration up to 5% to prevent the cells from sticking on the side of the tubes. In addition, an attempt to detach the cells using trypsin or gentle scrubbing from the plate was also unsuccessful, resulting in damaged and non-viable cells. Using carp head kidney however, Joerink et al. (2006) were able not only to recover the sorted cells, but also culture the cells for several days and subsequently detach the cells for further characterisation by flow cytometry and for functional experiments (Joerink et al., 2006).

To further differentiate the cell types in the HKL and PTL populations, light microscopic cytochemistry was conducted. Peroxidase activity was detected in both HKL and PTL populations with different intensities. The peroxidase positive granular cells of HKL and PTL were identified as neutrophils. Peroxidase has been observed in the neutrophils of teleosts, including sturgeon, *Acipenser* sp. (Hine and Wain, 1988), sea bass, *Dicentrarchus labrax* (Do Vale et al., 2002) and rainbow trout, *Oncorhynchus mykiss* (Afonso et al., 1997). Some neutrophils were strongly peroxidase positive with a more intense brown colour but some were weakly positive with light brown staining. This evidence may be interpreted in terms of the maturity of the cells. Circulating neutrophils found in European eel, *Anguilla anguilla*, were peroxidase positive but their precursors in head kidney were negative in the latter stages weakly positive (Kreutzmann and Nasev, 1979). The presence of hydrolases of non-specific esterase was found in HKL and PTL populations. Monocytes and macrophages stained brown under α-naphthyl esterase (ANAE). Studies have showed that the diffuse reaction product of non-specific esterase in the cytoplasm of monocytes/macrophages can be readily distinguished from the discrete granular non-specific esterase staining in granulocytes and lymphocytes of fish leucocytes (Tavares-Dias, 2006). The esterase-positive cells identified in both HKL and PTL populations agree with the type of cells considered to be monocytes/macrophages in Diff Quick-stained preparations.

By morphology, barramundi HKL populations were dominated by lymphocytes, with small monocytes and neutrophils, whilst PTL comprised mainly large lymphocytes, monocytes and macrophages and to lesser extent neutrophils. Further ultrastructural examination by electron microscopy is required to corroborate the identities of the cell types in these populations. However, the present data do indicate that the peritoneal populations and those isolated from head kidney are different.

In light of these results, the capabilities of the two populations to respond to various PAMP immunostimulants was investigated, as they may be expected to respond differently, reflecting the differing population structures. The present results support the hypothesis that, as these two cell populations are structurally distinct, their abilities to produce radical oxygen species as part of host defence mechanism against invading pathogens is also different. HKL had a lower response to stimulation by LPS than PTL, with the response in PTL 9-fold higher than in HKL. Higher concentrations of LPS suppressed the response by 50% in the PTL. The LPS receptors are mainly found in monocytes and macrophages in mammals (Morrison et al., 1992) and probably in fish (Iliev et al., 2005b). In mammals, LPS binds to CD14 which is facilitated by lipopolysaccharide binding protein (LBP) expressed on monocytes and macrophages (Tapping and Tobias, 1997). A possible absence of several proteins including LBP involved in LPS signalling pathway and possibly different pathway mechanisms in fish, results in fish being more resistant to the toxic effects of LPS (Iliev et al., 2005b). Stimulation by LPS in fish thus requires higher concentrations up to 1000-fold higher than the concentrations required to stimulate mammalian cells (Kataoka et al., 2002; Iliev et al., 2005a). The lack of LPS membrane receptors on fish lymphocytes, which were found to be abundant in barramundi HKL populations, may explain the inability of HKL to up-regulate radical oxygen species production significantly *in vitro* in response to LPS. Other studies have also revealed that upon stimulation with LPS, head kidney derived leucocytes of goldfish (Neumann et al., 1998), gilthead seabream (Mulero et al., 1998) and sea bass (Sarmento et al., 2004) demonstrated an enhanced respiratory burst activity.

Peptidoglycan (PTG) is a complex outer membrane carbohydrate of Gram-positive bacteria that has been shown to stimulate the production of inflammatory mediators *in vitro*. PTG can induce strong antibacterial response, and activates monocytes, macrophages and B-lymphocytes in mammals (Aderem and Underhill, 1999). In this study, stimulation of HKL and PTL by PTG did not have a significant effect on their ability to produce ROS. Injection of 1 μg mL^{-1} PTG into Japanese flounder resulted in activation of immune response which was demonstrated by increase in production of intracellular superoxide anion and resistance to bacterial challenge (Kono and Sakai, 2001; Kono et al., 2003). This perhaps indicates that other factors in the fish that are not present in culture are required for PTG to activate fish macrophages.

Priming the cells with LPS over different periods of time, followed by triggering with PMA, resulted in increase of the respiratory burst

activities of PTL by 2- to 7-fold compared to triggering un-primed cells. There was, however, an optimum time for priming with cells primed for 6 h having significantly higher RB following triggering than those primed for 3 h or 24 h, perhaps indicating that PTL cells became exhausted after extended exposure. HKL could also be successfully primed by exposure to both concentrations of LPS employed, however the effect of time was different. Indeed, whilst differences were only marginally significant, higher RB was recorded from cells primed for 24 h with LPS compared to 3 and 6 h. This may reflect the fact that HKL cells had not been stimulated prior to collection. In contrast, the PTL have been harvested following stimulation of the peritoneum with casein. Whilst casein is not a PAMP and merely serves to mildly irritate the peritoneum to induce a mild inflammatory response, it may be that this is additional stimulation, followed by extended priming with a high dose of LPS then triggering, may have exhausted the supply of raw materials such as myeloperoxidase to sufficient extent to reduce the cells' respiratory burst activity. A similar pattern of reduction in respiratory burst activity caused by longer incubation time was also measured by flow cytometry in PTL populations, with a marked depletion of percentage of green fluorescent cells after 45 min stimulation, whilst the HKL demonstrated an increase after that time. Altogether, the results reported here show that HKL required longer incubation time to achieve an optimum production of ROS (24 h as opposed to 6 h for PTL), allowing the cells to mature, differentiate and respond to the stimulating agent. In contrast, the PTL cells are already mature, active cells, sent to respond to irritation in the peritoneum. These cells are ready to respond immediately to stimuli that they encounter, and respond with rapid and high production of ROS. Extended exposure to stimuli, however, appears to exhaust the cells, reducing their ability to respond.

The luminol-enhanced chemiluminescence assay gives an indication of the total redox potential generated by the total cell population cells stimulation. However, this activity is derived from a number of reactive oxygen and nitrogen derivatives. To try to determine what proportion of the cells resulted in this activity, we assayed hydrogen peroxide production using flow cytometry. Fluorescence intensity as a result of peroxide production was similar in both populations of cells, but the kinetics differed, with PTL switching on faster (45 min to peak activity) than HKL (60 min to peak activity). It may be that the differences recorded in the chemiluminescence assays resulted from different reactive oxygen derivatives, with more superoxide, singlet oxygen or other radicals produced by PTL cells that are detected by luminol, but do not induce fluorescence in DHR123.

Production of nitric oxide as a result of respiratory burst in phagocytic cells is more complex. A pilot investigation revealed that a combination of LPS and IFN-γ was required for maximal induction of NO production in both HKL and PTL. The specificity of the assay was confirmed by a significant reduction in production of inducible nitric oxide after treatment with diphenyleneiodonium chloride (DPI), a specific inhibitor of inducible nitric oxide synthase in both HKL and PTL. However, the DPI inhibited the NO production to a level lower than that seen in unstimulated controls. This suggests the iNOS is not the only target of DPI in barramundi leucocytes, and that constitutively expressed NOS such as eNOS may also have been inhibited, thus reducing constitutively produced NO in these cells. Alternatively, the cells used in this assay may have been induced by other factors, increasing iNOS expression in the control cells as well. This seems a possibility as high induction of NOS was detected during the time course study after 3, 6 and 12 h induction. Similar low level induction or high background have been reported previously: The basal induction of European sea bass (*D. labrax*) head kidney leucocytes with MAF, LPS, or a combination of both stimuli failed to produce a detectable level of NO (Sarmento et al., 2004). This group suggested that it could be due to the fact that head kidney cells predominantly consisted of undifferentiated cells that would require some further stimulation to mature to perform their potential function, including production of NO (Sarmento et al., 2004). The concept of maturation is corroborated by other studies; the head kidney leucocytes of common carp, cultured for 6 days and stimulated with or without LPS for 18 h showed a significant increase in NO production relative to newly isolated cells (Belosevic et al., 2006; Joerink et al., 2006). They observed that goldfish (*Carassius auratus*) head kidney leucocytes comprise three distinct macrophage sub-populations, which were different in their ability to produce nitric oxide. It was identified that the population that contained macrophage-like cells was the potent producer, whilst the other two populations demonstrated little or no production of NO when stimulated with MAF, LPS or both (Belosevic et al., 2006; Joerink et al., 2006). Such differences were thought to be due to differences in maturation stages of the cells, hence monocytes need to differentiate into mature macrophages to acquire capacity to produce NO. A relatively high NO production level of unstimulated HKL and PTL observed in the current study could be caused by tissue stress or injury during preparation of the assay. These results are in accordance with the observations by Sarmento et al. (2004), who also found that the untreated cells showed high production of superoxide anion relative to the treated cells.

In conclusion, this study shows that both barramundi HKL and PTL can be stimulated to enhance their respiratory burst activity by LPS and PMA, but not PTG. The PTL mainly consisted of mature cells like macrophages and consequently required lower stimulant concentration and incubation time compared to HKL, which mostly contained immature cells including lymphocytes and monocytes. Their distinction in terms of population structure and morphological characteristics was thus confirmed to some degree by their functional dissimilarities in stimulation of ROS production. Care should be exercised when using leucocyte populations in *in vitro* host–pathogen interaction studies, as freshly isolated head kidney cells do not operate at their full biochemical potential in terms of their ability to enter respiratory burst and to produce reactive microbicidal products. Such studies may lead to false interpretations of pathogens' strategies for host colonisation.

Acknowledgements

Reiny Tumbol was supported during her doctoral study by an Ausaid Scholarship. Research consumables were supported through the University of Queensland URG. We are grateful to Geoff Osborne and Virginia Nink of the Queensland Brain Institute for their expert assistance with the flow cytometry, and to the Australian Institute for Biotechnology and Nanotechnology for use of the Cytospin centrifuge.

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Differing cell population structure reflects differing activity of Percoll-separated pronephros and peritoneal leucocytes from barramundi (Lates calcarifer)

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