



THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Research Commons

<http://waikato.researchgateway.ac.nz/>

Research Commons at the University of Waikato

Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

**Flow Cytometric Enumeration of the Blood Cells of Rainbow Trout
(*Oncorhynchus mykiss*) and New Zealand Freshwater Crayfish
(*Paranephrops planifrons*)**

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science in Biological Sciences

at

The University of Waikato

by

SEAN CHARLES TAYLOR

Department of Biological Sciences

2009



THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Abstract

The aim of this study was to develop flow cytometric (FC) methods to enumerate rainbow trout (*Oncorhynchus mykiss*) whole blood cells and New Zealand freshwater crayfish (*Paranephrops planifrons*) haemocytes as non-lethal endpoints in the evaluation of physiological status.

In the FC method development for rainbow trout, heparin was found to be superior to neutralised EDTA as a blood anticoagulant, because the use of EDTA resulted in significant lysis and shrinkage of erythrocytes. Leishman's-Giemsa and May Grunwald-Giemsa yielded comparable differential staining of leukocytes, and were superior to Wright-Giemsa staining. Morphological ambiguity between thrombocytes and lymphocytes in smears could not be resolved using Romanowsky or cytochemical staining. Use of FC was demonstrated to be a rapid, more accurate alternative to manual total cell counting procedures. Phosphate-buffered saline (PBS) was found to be superior to IsoTon[®] II as a FC sheath fluid; IsoTon[®] II induced lysis of erythrocytes. Characterisation of fish blood cell types and differentiation of leukocytes using FC could be achieved using 50 nM concentrations of the fluorescent lipophilic dye 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3)), but inconsistent fluorescent behaviour exhibited by thrombocytes between specimens prevented clear resolution of these cells from erythrocytes and lymphocytes. Higher concentrations (>200 nM) of DiOC₆(3) did not enhance resolution and became cytotoxic, particularly to leukocytes. Resolution between thrombocytes and lymphocytes could only be achieved with a fluorescent-labelled thrombocyte monoclonal antibody (mAb). The results suggest that the application of FC and mAb to fish blood cells is the most accurate approach to differential counting of leukocytes.

The second FC method objectively characterised and enumerated New Zealand freshwater crayfish haemocytes. Haemocyte populations were isolated by FC sorting based on differential light scatter properties, followed by morphological characterisation by light microscopy and software image analysis. Cells were identified as hyaline, semi-granular and granular haemocytes based on established invertebrate haemocyte classification. A characteristic decrease in

nuclear size and increase in granularity between the hyaline and granular cells, and the eccentric location of nuclei in granular cells were also observed. The granulocyte subpopulations were observed to possess varying degrees of granularity. The developed methodology was used to perform total and differential haemocyte counts from three lake crayfish populations and between wild and captive specimens. Differences in total and differential haemocyte counts were not observed between wild populations. However, specimens held in captivity for 14 d exhibited a significant 63% reduction in total haemocyte count, while the relative haemocyte proportions remained the same. These results demonstrate the utility of this method for the investigation of sub-acute stressor effects in selected decapod crustacea.

Acknowledgments

They say that no good deed goes unpunished, so here goes...

First and foremost, many, many thanks to my supervisors, Dr Nick Ling and Dr Mike Landman, for your supportive guidance up what turned out to be quite a winding creek, particularly when I lost my paddle. I really appreciate your encouragement, enthusiasm and expertise in what was pretty much uncharted water for me (and I'll stop there with the riverine analogies). Good luck over the deetch, Mike – been a pleasure fushing with you.

I'm grateful for the opportunity and sponsorship provided by the New Zealand Forestry Research Institute (Scion) to undertake this project, particularly to Dr Trevor Stuthridge, Sustainable Design Group Manager, for your support.

Thanks to Mark Sherburn, Lloyd Gledhill and the team at New Zealand Fish and Game, Eastern Region, for supplying us with the trout. Crayfish research was funded by Environment Bay of Plenty and through a New Zealand Foundation for Research Science and Technology (FRST) contract with Scion. We are grateful for the field assistance of Jeroen Brijs during specimen collection, and Iwi consultation with Roku Mihinui and Hera Smith of the Te Arawa Lakes Trust, facilitated by Tupara Morrison (Scion). *Kia ora koutou.*

On the technical front, hats off to the team at BD Biosciences – Graeme Chapman, Andrew Mansfield, Lorraine Pal and Robert Sleiman. Your collective training, advice and support with the flow cytometer capability are much appreciated. Thanks also to Alan Crowe at Lakes District Health Board laboratory, for letting me loose on their osmometer to wade through my plasma samples, and for shouting me coffee on those long days at the bench wearing a funny coat.

Sincere thanks to Dr. Bernd Köllner (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Germany), for the generous donation of the trout antibodies.

Other sets of eyes and the voices of experience are always good to have around when it comes to this sort of thing, so cheers to Marie Dennis, Gerty Gielen and Sarah Addison for the proof-reading and the formatting tutorials.

A big thank you to my extended family for all the moral support, and especially to Cecilia for stepping in to help us out on the home front when we needed it most during the last lap.

And to Audrey, Steven and Adam (and our little surprise), thank you always for being my compass.

For Mom and Dad

Table of Contents

Abstract	ii
Acknowledgments	iv
Table of Contents	vii
List of Figures	x
List of Tables	xiii
List of Plates	xiv
Chapter One: General Introduction	1
1.1 Project background	2
1.2 Challenges in haematological assessment of fish and crustacea	2
1.3 Flow cytometry	4
1.4 FC in the study of aquatic species	8
1.5 Project objectives	9
1.6 Animal ethics statement	9
1.7 References	9
Chapter Two: Flow cytometric analysis of the peripheral blood of rainbow trout (<i>Oncorhynchus mykiss</i>)	13
2.1 Introduction	14
2.2 Materials and methods	18
2.2.1 Animals	18
2.2.2 Sampling protocol	18
2.2.3 Traditional haematology	19
2.2.3.1 Haematocrit	19
2.2.3.2 Haemoglobin	19
2.2.3.3 Total red blood cell count	20
2.2.3.4 Total leukocyte count	20
2.2.3.5 Differential leukocyte count	21
2.2.3.6 Cytochemistry	22
2.2.3.7 Photomicroscopy	22
2.2.4 Flow cytometry	23
2.2.4.1 Flow cytometer	23
2.2.4.2 Tube preparation	23
2.2.4.3 Fluorescent dye staining	24

2.2.4.4 Monoclonal antibody and fluorescent conjugate labelling.....	25
2.2.4.5 Instrument parameters	25
2.2.4.6 Cell counting	26
2.2.4.7 Cell sorting	26
2.2.5 Experimental protocol	27
2.2.5.1 Anticoagulant evaluation.....	27
2.2.5.2 Diluent compatibility evaluation	28
2.2.5.3 Fluorescent staining evaluation	29
2.2.5.4 Flow cytometric count validations with fluorescent dye only.....	29
2.2.5.5 Flow cytometric count validations with fluorescent dye and thrombocyte antibody.....	30
2.2.6 Statistical analyses.....	30
2.3 Results	32
2.3.1 Anticoagulant evaluation.....	32
2.3.2 Diluent compatibility evaluation	36
2.3.3 Characterisation of blood cell types and cytochemical staining	36
2.3.3.1 Erythrocytes.....	36
2.3.3.2 Granulocytes.....	39
2.3.3.3 Lymphocytes	39
2.3.3.4 Thrombocytes.....	39
2.3.3.5 Monocytes	39
2.3.4 Fluorescent staining evaluation	40
2.3.5 Flow cytometric count validations with fluorescent dye only	47
2.3.6 Flow cytometric count validations with fluorescent dye and thrombocyte antibody.....	50
2.4 Discussion.....	53
2.5 References	60

Chapter Three: Flow cytometric characterisation of freshwater crayfish

haemocytes for the examination of physiological status in wild and captive

animals.....	68
3.1 Introduction	69
3.2 Materials and methods.....	70
3.2.1 Animals	70
3.2.2 Haemolymph sampling procedure.....	71

3.2.3 Preliminary flow cytometric sample evaluation.....	71
3.2.4 Sorting and characterisation of haemocytes	72
3.2.5 Hemocyte evaluation in wild and captive crayfish.....	73
3.2.6 Statistics.....	74
3.3 Results	74
3.4 Discussion.....	82
3.5 References	84
Chapter Four: General Conclusions and Recommendations.....	89
4.1 Conclusions	90
4.2 Recommendations	91
4.3 References	92

List of Figures

Figure 1.1. BD FACSVantage™SE flow cytometer configuration, indicating fluidics, laser light paths, optical bench configuration, bandpass filters, photomultiplier detector channel assignments (FSC, SSC, FL1 through FL8) and equivalent chromophores (in parentheses), and sorting module function.

Figure 2.1. Blood parameters (haematocrit (A) and plasma haemoglobin (B)) of rainbow trout blood ($n = 10$) incubated with 500 i.u. mL^{-1} heparin and 4 mg mL^{-1} EDTA for 120 min. Asterisks indicate significant differences ($P < 0.1$, Tukey's HSD test). Values are mean \pm SEM.

Figure 2.2. Mean erythrocyte cell length (A) and width (B) (\pm SEM, $n = 50$) for blood treated with 500 i.u. mL^{-1} ammonium heparin and 4 mg mL^{-1} neutralised EDTA. Asterisks indicate significant differences ($P < 0.05$, paired T- test).

Figure 2.3. Flow cytometric side scatter (SSC) vs. 3,3'-dihexyloxacarbocyanine iodide fluorescence (FITC-A) dot plots for rainbow trout blood treated with 25 to 1000 nM dye. In 50% of samples, five cell populations (P1 to P5) were observed at 25 nM dosage (A). P1, P2 and P3 shifted to notably higher fluorescence intensities with increasing dye concentration, while P4 and P5 only exhibited slight increases (B). P3 and P4 were observed to merge at ≈ 500 nM (C). In some specimens, P3 did not appear to be resolved, irrespective of dye dosage (D, E & F).

Figure 2.4. Cytotoxic effects of increased dosage of 3,3'-dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$) on rainbow trout erythrocytes (A) and leukocytes (B), indicated by Sytox® Red positivity using flow cytometry. Significant toxic effects ($P < 0.05$, Kruskal-Wallis multiple comparisons test) are indicated with asterisks. $n = 10$ in all tests. Values are mean \pm SEM.

Figure 2.5. Flow cytometric side scatter (SSC) vs. 3,3'-dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$) fluorescence (FITC-A) dot plots of rainbow trout blood cells treated with 50 to 1000 nM dye, indicating erythrocytes (e), thrombocytes (t), lymphocytes (l), granulocytes (g) and Sytox® Red-positive events (non-viable cells) indicated in green.

Figure 2.6. Flow cytometric side scatter (SSC) vs. forward scatter (FSC) dot plots of rainbow trout blood cells treated with 50 to 1000 nM 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3)), with erythrocytes indicated in red, leukocytes in blue and Sytox[®] Red-positive events (non-viable cells) indicated in green.

Figure 2.7. Mean (\pm SEM) total erythrocyte count (RBC L⁻¹) on rainbow trout whole blood using manual methods and flow cytometry with 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3)). Significant differences in counts ($P < 0.05$, paired T-test) are indicated with an asterisk.

Figure 2.8. Flow cytometric side scatter (SSC-A) vs. 3,3'-dihydroxyoxycarbocyanine iodide (FITC-A) fluorescence dot plot of rainbow trout blood, showing poor resolution of thrombocytes (T) from erythrocytes (RBC) at 50 nM dye, consistent across an entire sample subset ($n = 10$).

Figure 2.9. Flow cytometric dot plots of 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3)) fluorescence (FITC-A) and scatter data showing shifts in fluorescence intensity of thrombocytes (blue) in three specimens, at fixed dye dosage (50 nM) (A, C & E). The side scatter (SSC) vs. forward scatter (FSC) data for the gated leukocytes only (B, D & F for the three specimens, respectively) indicate trends in FSC similar to those observed in the fluorescence data.

Figure 2.10. Mean percentage coefficient of variation (%CV) data for gated event parameters of leukocytes labelled with thrombocyte monoclonal antibody 42-allophycocyanin conjugate and counterstained with 50 nM 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3)). Thrombocytes exhibited the most variation in terms of size (FSC), intracellular complexity (SSC) and DiOC₆(3) fluorescence.

Figure 2.11. Comparative total erythrocyte counts (RBC L⁻¹; A) and total leukocyte counts (WBC L⁻¹; B) on rainbow trout ($n = 10$) using manual methods, 50 nM 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3)) and thrombocyte mAb +allophycocyanin conjugate+DiOC₆(3) counterstaining procedures. Significant differences ($P < 0.05$, Tukey's HSD post-hoc test) are indicated with asterisks. Values are mean \pm SEM.

Figure 2.12. Differential leukocyte counts (DWBC) on rainbow trout (n = 10) using manual methods, 50 nM 3,3'-dihexyloxacarboyanine iodide (DiOC₆(3)) and thrombocyte mAb+allophycocyanin conjugate+DiOC₆(3) counterstaining procedures. Significant differences (P < 0.05, Wilcoxon matched pairs test) are indicated with asterisks. Values are mean ± SEM.

Figure 3.1. Gating procedure for haemocyte counts on 3,3'-dihexyloxacarboyanine iodide (DiOC₆(3))-stained hemolymph samples. A) Fluorescent event (FL1) gate, B) forward scatter (FSC) subset of FL1 gate and C) side scatter (SSC) subset of the FSC gate.

Figure 3.2. Representative forward scatter (FSC) vs. side scatter (SSC) data plots of hemolymph showing A) all cellular events, B) gating of individual hyaline (H), semigranular (SG) and granular (G) hemocyte populations and C) “20% probability” contour plots of hemocyte gates.

Figure 3.3. Comparative total hemocyte counts of A) wild lake specimens and B) wild vs. 14 d-captive specimens. Values are mean ± SEM.

Figure 3.4. Differential hemocyte counts in A) wild lake specimens and B) wild vs. 14 d-captive specimens. Columns are hyaline (dotted), semigranular (diagonal hatch) and granular (open) cells. Values are mean ± SEM.

List of Tables

Table 2.1. Mean (\pm SEM) blood parameters of samples diluted 1:3 with PBS and IsoTon[®] II (n = 10 for each) and incubated on ice for 120 min. Asterisks indicate significant ($P < 0.05$, Wilcoxon matched pairs test) measured parameter differences between diluents.

Table 2.2. Dimensions and cytochemical staining reactions of characterised rainbow trout blood cell types. – = negative, + = mildly positive, ++ = moderately positive, +++ = very positive, no result = not tested.

Table 3.1. Mean (\pm SEM) length (l) and diameter (d) for sorted hemocytes and nuclei (n = 30). Asterisks indicate significant differences ($P < 0.05$, Tukey's post-hoc test) in cell area and nucleocytoplasmic ratio between cell types.

List of Plates

Plate 1.1. BD FACSVantage™ SE flow cytometer.

Plate 2.1. Staining characteristics of blood cells sampled in heparin (A, C & E) and EDTA (B, D & F) and stained with Leishman's-Giemsa (A & B), May-Grünwald-Giemsa (C & D) and Wright's-Giemsa (E & F) formulations.

Plate 2.2. Characterisation of rainbow trout blood cell types (arrowed). Staining with Leishman's-Giemsa revealed erythrocytes (A), immature erythrocytes (B), lymphocytes (D), granulocytes (E) and spindle-shaped and ovoid to round thrombocytes (F,G). Brilliant Cresyl Blue staining confirmed the presence of immature erythrocyte ribonucleoprotein (C). Cytochemical staining revealed positive response of granulocytes to Sudan Black B (I), Periodic acid-Schiff (J), myeloperoxidase (K) and alkaline phosphatase (L) tests. Staining with Toluidine Blue at pH 9 (H) resulted in leukocyte staining comparable to LG.

Plate 2.3. Cytocentrifuge preparations of sorted rainbow trout blood cells stained with Leishman's-Giemsa, including erythrocytes (A, B & C), thrombocytes (D), lymphocytes (E) and granulocytes (G). Lymphocytes (L) and thrombocytes (T) occasionally demonstrated morphological ambiguity (F). Granulocytes, which exhibited myeloperoxidase positivity in smears, yielded negative results in cytocentrifuge preparations (H).

Plate 3.1. Sorted haemocytes from predefined populations stained with Leishman's-Giemsa. A) Hyaline (H), showing variation in nuclear size and staining, B) semigranular (SG), showing variation in nuclear size and staining and cytoplasmic granularity and C) granular (G), showing variation in intensity of eosinophilic staining of granules. All figures x 1000.

Chapter One

General Introduction

1.1 Project background

Declining water quality in freshwater lakes in the Bay of Plenty region, New Zealand, has been the focus of targeted causal and remedial research in recent years. Issues related to changes in trophic status of receiving environments have prompted local and regional authorities to fund scientific and commercial evaluation of short, medium and long-term causes and effects, and remedial solutions. Monitoring of the physiological status of fish and invertebrates in impacted environments and those exposed to remedial treatments, such as alum (aluminium sulphate), and modified clays and zeolites has been integral to ongoing environmental impact assessment. The use of such biological indicators in evaluation of aquatic system health has been well described (Adams, 1990; McCarthy and Shugart, 1990). Several ecotoxicological endpoints such as metal bioaccumulation and endocrine disruption can only be determined using methods that inevitably involve removal and sacrifice of animals. The use of haematology has been mooted as a non-lethal physiological monitoring procedure that may be performed regularly, on statistically robust samples, on a variety of species representative of ecological communities.

This thesis describes the development of instrumental flow cytometric methods for total and differential counting of peripheral blood cells and haemocytes of rainbow trout (*Oncorhynchus mykiss*) and the New Zealand freshwater crayfish (Northern *koura*, *Paranephrops planifrons*) respectively, as models for the physiological evaluation of piscine and invertebrate sentinel species in freshwater environments.

1.2 Challenges in haematological assessment of fish and crustacea

Abnormal changes in total and differential blood cell counts in fish, such as anaemia, leukopaenia, leukocytosis and thrombocytopaenia, may result from diseases, but may also indicate stress, toxic exposure, hypoxia and changes in reproductive status (Campbell and Ellis, 2007). Haemocyte counts in crustacea may similarly vary in response to microbial infection, environmental stress and endocrine activity (Johansson et al., 2000). The haematology of man is well

understood, and reference ranges and normal values for diagnostic assessment of health, that account for known variables such as age, sex, occupation, body-build, diet and geographic location may be standardised for populations of interest (Lewis, 2001). Pathological effects and response to treatment may only be monitored objectively using haematology if these variables are recognised and accounted for. Given the diversity of species, and the highly variable nature of the physiology and ecology of fish and crustacea, generalisations about reference ranges and normal values for haematological parameters cannot be made (Hose et al., 1990; Hrubec and Smith, 2000).

Lewis (2001), on human haematology, stated: “A number of factors affect haematological values in apparent health. The technique and timing of collection, transport and storage of specimens and differences in the subject’s posture when the sample is taken and whether ambulant or confined to bed, may have an effect...Variation in the analytical methods used may also affect the measurements.” This generalised statement has similar relevance in the assessment of multiple aquatic species in a variety of environments. Inherent problems associated with such a strategy are a) a lack of physiological knowledge related to the individual species, and how this may be affected by various intrinsic (organism) and extrinsic (environmental) factors and b) the imprecise, time-consuming and labour-intensive nature of manual haematological enumeration procedures.

The sensitivity of fish and crustacea to chronic and acute stressors, and their responses to changes in the aquatic environment, has been extensively researched and described at generic and species levels (Roche and Boge, 1996; Le Moullac and Haffner, 2000), and herein lies the value of their use as biological indicators. However, the physiological response of an animal to the procedure of blood or haemolymph sampling may potentially influence the detection of a primary stressor. If the effects of handling a fish for as little as 20 seconds, resulting in significant swelling of red blood cells (Campbell and Ellis, 2007), and the rapid clotting processes of crustacean haemolymph (Kwok and Tobe, 2006) cannot be inhibited, objective diagnosis may be compromised or negated. Sampling issues may sometimes be resolved with the appropriate use of

anaesthesia and suitable choice of anticoagulant formulations. A more serious inherent problem in quantitative blood and haemolymph assessments is the use of manual cell counting and differentiation procedures. Due to the nucleated nature of red blood cells (erythrocytes) in fish, white blood cells (leukocytes), which serve as an indicator of health, cannot be distinguished using automated cell counting procedures without lysis of erythrocytes (see Section 2.1), and are usually manually counted using a haemocytometer. Differential leukocyte and haemocyte enumerations, which also serve as health indicators, are generally performed either on stained smears or with a haemocytometer in fish and crustacea, respectively. The disadvantage of manual enumeration is the statistical limitation associated with counting between 100 to 200 cells, the typical range in differential leukocyte procedures (Bain and Bates, 2001). For rare cell types, this results in high coefficient of variation (CV) values (standard deviation / mean x 100%). Minimisation of CV, a measure of analytical precision, is achieved by counting a greater number of cells, which may be impractical on large numbers of samples. It follows that manual cell counting techniques are inherently imprecise (Shapiro, 2003b).

1.3 Flow cytometry

Flow cytometry is an instrumental technique in which a stream of suspended particles is interrogated by one or more lasers. Particles are analysed and differentiated on the basis of their light-scattering properties, auto- or labelled fluorescence, or a combination of both. Forward scatter (FSC) intensity is related to the refractive index of a particle and its cross-sectional area, and 90° scatter (side scatter, SSC) intensity is generally related to surface or internal irregularity or granularity of a particle (Givan, 1994a). In biological studies, cellular autofluorescence and the use of fluorescent dyes and monoclonal antibodies (mAb) to label cells provide additional parameters for detection and discrimination. The major advantages of FC technology are the ability to differentiate and enumerate several thousands of particles per second, and to physically sort multiple populations simultaneously into collection vessels. In haematological applications, the capability to obtain accurate and precise total and

differential blood counts on so many more cells than practically achievable with manual methods, in a fraction of the time, is thus dependent only on the ability to accurately discriminate between cell types.

After dispersion in appropriate media, cells are aspirated into the centre of a stream of water or buffered saline, known as the sheath fluid. The cell suspension is hydrodynamically focussed to form a narrow core in the sheath fluid stream as it is pneumatically aspirated through a porcelain nozzle (50 to 400 μm orifice diameter), to optimise the uniform passage of one particle at a time (Givan, 1994b). The stream is intercepted by one or more monochromatic lasers. Instruments are available which may incorporate multiple lasers that supply excitation at 488 nm (blue light), at 633 nm (red light) and 325 nm (ultra-violet; UV), allowing for multi-colour fluorescence evaluations (Ormerod, 1999). Photomultiplier tubes serve as detectors, with appropriate dichroic mirrors and bandwidth filters placed in the beam path of SSC light, to isolate and measure the fluorescence parameters of interest. The instrument used in this study (Plate 1.1), and a schematic of the laser configuration, sample and fluid stream, bandpass filters and equivalent chromophores, detector configuration and sorting function (Fig. 1.1) are depicted below.



Plate 1.1. BD FACSVantage™ SE flow cytometer.

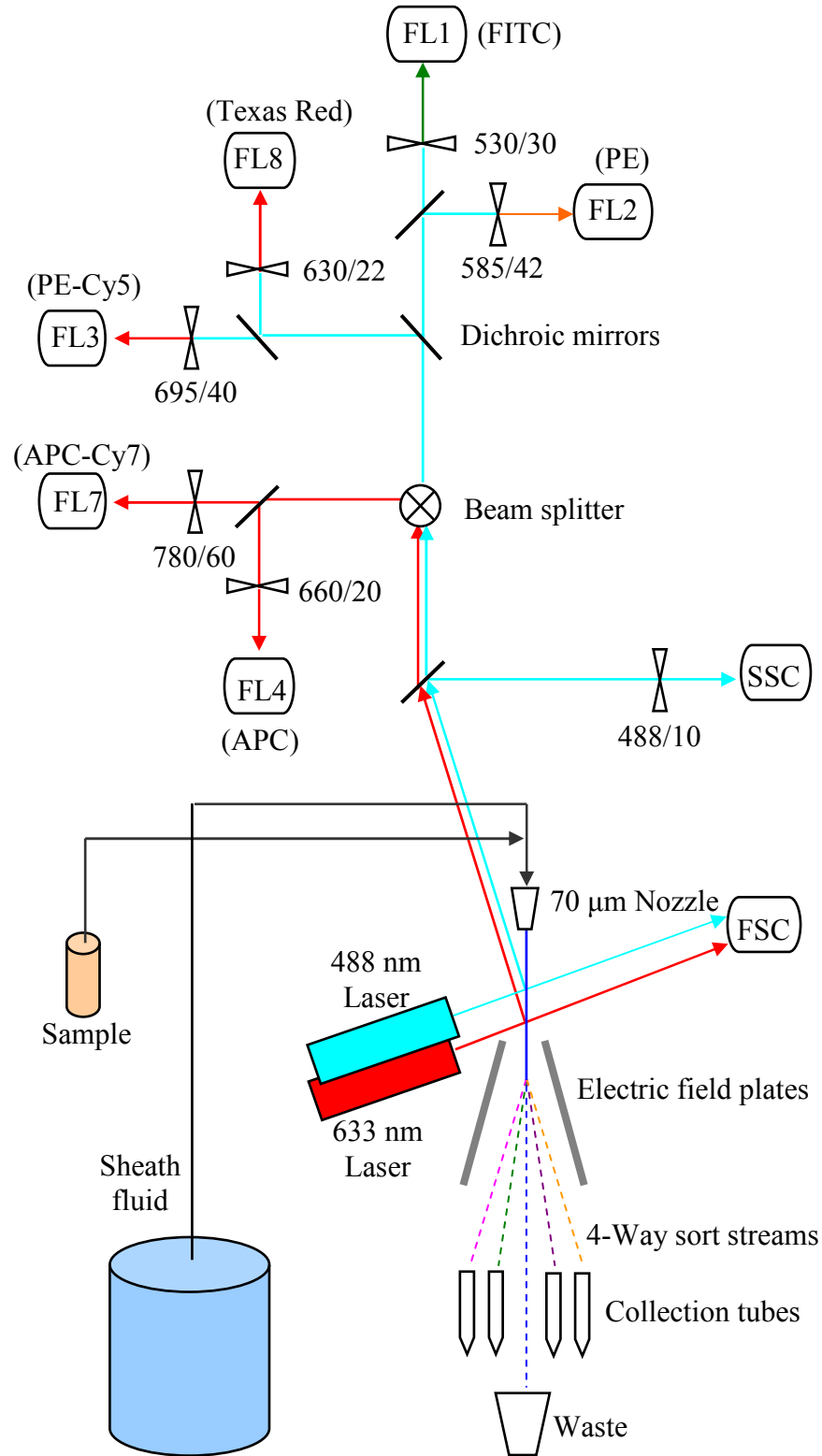


Figure 1.1. BD FACSVantage™ SE flow cytometer configuration, indicating fluidics, laser light paths, optical bench configuration, bandpass filters, photomultiplier detector channel assignments (FSC, SSC, FL1 through FL8) and equivalent chromophores (in parentheses), and sorting module function.

The electrical signals from the photomultipliers are then modulated using linear or logarithmic amplification. Scatter parameters are typically viewed in linear mode and fluorescence parameters in logarithmic mode, based on the range of output intensities of these parameters (Givan, 1994b). Amplified signals are subjected to analog-to-digital conversion and viewed on-screen as either single-parameter histograms or bivariate histograms, also referred to as cytograms. Bivariate cytograms may be presented as dot plots or contour plots (Shapiro, 2003b). Use of bivariate cytograms facilitates discrimination of clusters, or populations, of events. Unwanted events, such as those arising from debris or instrumental noise, are excluded by “triggering” or “thresholding” on one or more parameters. This defines the limit below which events are not counted, and is typically employed on the FSC signal, i.e. on the basis of particle size (Givan, 1994b).

The cell sorting mechanism employed by most commercially available instruments is droplet sorting. The sheath fluid/sample stream is broken into droplets, using a piezoelectric transducer, as it passes through the nozzle. Droplets, optimally containing a single cell of interest, are electrostatically charged and deflected into collection vessels as they pass through an electric field (Shapiro, 2003a). The time elapsed between the passage of a cell through the laser intercept point to the time it is located in the last attached drop of the stream (the break-off point) is referred to as the drop delay, which is accurately determined to facilitate the correct charge property assignment to the droplet as it enters the electric field (Carter and Ormerod, 2000). As sorted cells are exposed to a proportionately high concentration of sheath fluid in the collection vessel for the duration of the sort, compatibility of the cells with the sheath fluid is critical, particularly if functional analysis is to be performed after recovery. Compatibility of the cells with fluorescent stain or antibodies is similarly important. Some cells lose viability through the sorting process while others appear to be unaffected (Givan, 2001).

1.4 Flow cytometry in the study of aquatic species

The use of FC in the study of fish blood cells and crustacean haemocytes is not new. The technique has been applied in the qualitative evaluation of immune response, phagocytosis and partial leukocyte differentiation in rainbow trout (Morgan et al., 1993; Chilmonczyk and Monge, 1999; Kfoury Jr et al., 1999; Scharsack et al., 2001). Partial differentiation of the leukocytes of other species, using fluorescent dye and monoclonal antibody (mAb) methods, have also been described (Ellsaesser et al., 1985; Inoue et al., 2002). Although correlation with traditional manual counts was generally reported to be good, only partial differentiation of leukocytes could be achieved without the use of mAb. Ellsaesser et al. (1985) used a fluorescent antibody to channel catfish (*Ictalurus punctatus*) immunoglobulin to demonstrate differentiation of most, but not all, lymphocytes from thrombocytes. Inoue et al. (2002) were able to achieve partial differentiation of carp (*Cyprinus carpio*) leukocytes using 3,3'-dihexyloxacarbocyanine (DiOC₆(3)), a non-specific lipophilic fluorescent dye, but were similarly unable to resolve thrombocytes and lymphocytes.

Characterisation and differentiation of marine prawn *Penaeus monodon* haemocytes using FSC and SSC has been demonstrated using FC (Owens and O'Neill, 1997; Yip and Wong, 2002). Flow cytometry has also been applied in the qualitative evaluation of the innate immunity of the freshwater crayfish *Procambarus zonangulus*, *in vitro* (Cardenas et al., 2000; Cardenas et al., 2004), and of differential haemocyte count variations associated with the moult cycle in male and female marine shrimps *Penaeus japonicus* (Sequeira et al., 1995). Xue et al. (2001) demonstrated the use of DiOC₆(3) in the characterisation and differential counting of vital marine oyster *Ostrea edulis* haemocytes.

1.5 Project objectives

The focus of this study was to develop methodologies for non-lethal, minimal-impact sampling of freshwater fish and crayfish that could subsequently be employed in determining species-specific physiological responses to environmental stressors. This was undertaken with the following objectives:

1. Develop blood and haemolymph sampling procedures that preserve the structural and morphological integrity of cells and thus the accuracy of enumeration;
2. Evaluate microscopic and FC procedures for the morphological and cytochemical characterisation of blood cells and haemocytes to facilitate differentiation;
3. Develop flow cytometric methods for the total and differential enumeration of cells in fish whole blood and crustacean haemolymph.

1.6 Animal ethics statement

All experiments in this study were approved by the Scion (Protocols 2007-006 and 2008-002) and University of Waikato (Protocol 738) Animal Ethics Committees.

1.7 References

Adams SM. 1990. Status and use of biological indicators for evaluating effects of stress on fish. *American Fisheries Society Symposium* **8**:1-8.

Bain BJ, Bates I. 2001. Basic Haematological Techniques. In: Lewis SM, Bain BJ, Bates I, editors. *Dacie and Lewis Practical Haematology*. 9th ed. Edinburgh: Churchill Livingstone. p 19-46.

Campbell TW, Ellis CK. 2007. Hematology of Fish. *Avian and Exotic Animal Hematology and Cytology*. Carlton: Blackwell Publishing Asia. p 93-111.

Cardenas W, Dankert JR, Jenkins JA. 2004. Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish and Shellfish Immunology* **17**:223-233.

Cardenas W, Jenkins JA, Dankert JR. 2000. A flow cytometric approach to the study of crustacean cellular immunity. *Journal of Invertebrate Pathology* **76**:112-119.

Carter N, Ormerod MG. 2000. Flow Sorting. In: Ormerod MG, editor. *Flow Cytometry: A Practical Approach*. Oxford: Oxford University Press. p 47-59.

Chilmonczyk S, Monge D. 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. *Fish and Shellfish Immunology* **9**:319-333.

Ellsaesser CF, Miller NW, Cuchens MA, Lobb CJ, Clem LW. 1985. Analysis of channel catfish peripheral blood leukocytes by bright-field microscopy and flow cytometry. *Transactions of the American Fisheries Society* **114**:279-285.

Givan AL. 1994a. Glossary. *Flow Cytometry: First Principles*. New York: Wiley-Liss. p 175-192.

Givan AL. 1994b. Instrumentation: Beyond the Black Box. *Flow Cytometry: First Principles*. New York: Wiley-Liss. p 15-40.

Givan AL. 2001. The Sorting of Cells. In: Givan AL, editor. *Flow Cytometry: First Principles*. New York: Wiley-Liss. p 159-174.

Hose JE, Martin GG, Gerard AS. 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *Biological Bulletin* **178**:33-45.

Hrubec TC, Smith SA. 2000. Hematology of fish. In: Feldman BF, Zinkl JG, Jain NC, editors. *Schalm's Veterinary Hematology*. 5th ed. Baltimore: Wiley-Blackwell. p 1120-1125.

- Inoue T, Moritomo T, Tamura Y, Mamiya S, Fujino H, Nakanishi T. 2002. A new method for fish leukocyte counting and partial differentiation by flow cytometry. *Fish and Shellfish Immunology* **13**:379-390.
- Johansson MW, Keyser P, Sritunyalucksana K, Soderhall K. 2000. Crustacean haemocytes and haematopoiesis. *Aquaculture* **191**:45-52.
- Kfoury Jr JR, Kuroda A, Nakayasu C, Fukuda H, Okamoto N. 1999. Analysis of rainbow trout peripheral blood leucocytes separated by flow cytometry cell sorting. *Fish Pathology* **34**:1-6.
- Kwok R, Tobe SS. 2006. Hemolymph clotting in crustaceans: implications for neuropeptide extraction from invertebrate hemolymph. *Peptides* **27**:590-596.
- Le Moullac G, Haffner P. 2000. Environmental factors affecting immune responses in crustacea. *Aquaculture* **191**:121-131.
- Lewis M. 2001. Reference ranges and normal values. In: Lewis M, Bain BJ, Bates I, editors. *Dacie and Lewis Practical Haematology*. 9th ed. Edinburgh: Churchill Livingstone. p 9-18.
- McCarthy JF, Shugart LR. 1990. Biological markers of environmental contamination. *Biomarkers of Environmental Contamination*. Boca Raton: Lewis Publishers. p 3-14.
- Morgan JAW, Pottinger TG, Rippon P. 1993. Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *Journal of Fish Biology* **42**:131-141.
- Ormerod MG. 1999. Instrumentation. *Flow Cytometry*. New York: Springer-Verlag. p 5-22.
- Owens L, O'Neill A. 1997. Use of a clinical flow cytometer for differential counts of prawn *Penaeus monodon* haemocytes. *Diseases of Aquatic Organisms* **31**:147-153.

Roche H, Boge G. 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Marine Environmental Research* **41**:27-43.

Scharsack JP, Steinhagen D, Leibold W, Rabe U, Korting W, Schuberth H-J. 2001. Flow cytometric analysis of mitogen-induced activation of rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Journal of Veterinary Medicine Series B* **48**:331-339.

Sequeira T, Vilanova M, Lobo-da-Cunha A, Baldaia L, Arala-Chaves M. 1995. Flow cytometric analysis of molt-related changes in hemocyte type in male and female *Penaeus japonicus*. *Biological Bulletin* **189**:376-380.

Shapiro HM. 2003a. Flow Sorting. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley & Sons. p 257-272.

Shapiro HM. 2003b. Overture. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley and Sons, Inc. p 1-60.

Xue Q-G, Renault T, Chilmonczyk S. 2001. Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. *Fish and Shellfish Immunology* **11**:557-567.

Yip ECH, Wong JTY. 2002. Fluorescence activated cell-sorting of haemocytes in Penaeid prawns. *Aquaculture* **204**:25-31.

Chapter Two

**Flow cytometric analysis of the peripheral blood of rainbow trout
(*Oncorhynchus mykiss*)**

2.1 Introduction

Fish live in intimate contact with the aquatic environment and their haematology may be useful in the assessment of stressor effects. For example, anaemias may be indicative of a range of physiological stressors including trauma, cutaneous ulceration, parasitism, nutritional deficiency, viral and bacterial septicaemia, environmental and bacterial toxins and renal or splenic disease (see reviews by Blaxhall, 1972; Campbell and Ellis, 2007b). More generally, haematology has been used to examine the physiological condition of wild and captive fish, both healthy and diseased, the effects of capture and confinement, seasonality and toxicant exposure (Bouck and Ball, 1966; Casillas and Smith, 1977; Barham *et al.*, 1980 Pickering and Pottinger, 1987; Woodward and Strange, 1987; Roche *et al.*, 1996; Benfey and Biron, 2000; Hoeger *et al.*, 2004; Rehulka and Adamec, 2004).

Fish possess two main blood cell types, the erythrocytes and leukocytes, but variations in the structure and function of blood cells may vary considerably between different groups and species (Fänge, 1992). Studies performed in the evaluation of the intrinsic effects of gender and age during spawning and the extrinsic effects of season and photoperiod on fish haematology (Pickering, 1986; Houston *et al.*, 1996; Valenzuela *et al.*, 2007) have demonstrated the importance of understanding temporal changes in blood parameters if these are to be used diagnostically. Many authors stress the importance and difficulty of establishing normal ranges for haematological parameters, but due to the number and morphological diversity of fish species, generalisations regarding the normal and pathological haematological states of fishes cannot be concluded (Hrubec and Smith, 2000; Campbell and Ellis, 2007b). Blaxhall and Daisley (1973) emphasised the importance of relating haematological information to experimental conditions, i.e. species, maturity, source, diet, temperature, and sampling and testing methods.

Haematological methods applied to fish are well-established and generally consistent with practices in human and veterinary haematology (Ellis, 1977). The tests are easy to perform but due to the sensitivity of fish to handling and sampling stress (Railo *et al.*, 1985; Campbell and Ellis, 2007), results need to be

interpreted with some degree of caution. Secondary stress responses include increased haematocrit (Hct), or percentage packed red blood cell volume, induced by haemoconcentration and adrenergic swelling of erythrocytes (Barton and Iwama, 1991; Campbell and Ellis, 2007b). Diagnosis of anaemia in fish is based on reduction in Hct, and in haemoglobin (Hb) concentration of blood (Campbell and Ellis, 2007b). An accurate red blood cell count (RBC) is intrinsically useful, but also facilitates calculation of the red cell indices, namely mean cell volume (MCV) and mean cell haemoglobin (MCH), which may additionally serve as a basis for classifying anaemias (Bain and Bates, 2001). Manual haemocytometric counting methods are inherently time-consuming, inaccurate and imprecise (Bain and Bates, 2001; Shapiro, 2003b) and have been largely superseded by instrumental counting techniques such as the Coulter counter and flow cytometry.

Leukocyte counts in fish are affected by stress, disease, nutritional disorders and the extrinsic and intrinsic factors similarly associated with effects on erythrocytes (Blaxhall and Daisley, 1973). The morphological, and to an extent functional, analogues of mammalian leukocytes (lymphocytes, neutrophils, eosinophils, basophils and monocytes) have been characterised based on well-established guidelines (Ellis, 1977; Rowley, 1990; Fange, 1992), but differences in morphology and cytochemical properties do exist between systematic groups. Teleostean lymphocytes respond to mitogens that are generally considered to be specific to mammalian lymphocytes; fish may possess the functional equivalents of lymphocytic T and B cells of higher vertebrates, as they demonstrate cell-mediated and humoral immune responses (Fange, 1992). Thrombocytes, the analogue of mammalian platelets, are responsible for blood clotting (Campbell and Ellis, 2007b) but unlike platelets, they are nucleated cells in fish. Their shape varies with stage of maturity and degree of reactivity, and may be round, oval, or spindle-shaped. Monocytes occur in low numbers and appear to be the primary phagocytic cell in fish (Ellis, 1977). Fish granulocyte morphology, distribution and function were extensively reviewed by Ainsworth (1992). The primary role of neutrophils, the predominant granulocyte, is phagocytosis, while the eosinophils have cytotoxic function and do not appear to participate in phagocytosis. Basophils occur only rarely in fishes and their function has yet to be objectively established.

A primary stress response in fish, to almost all forms of environmental stress, is the secretion of cortisol by the interrenal tissue (Donaldson, 1981). The release of cortisol results in lymphopaenia and neutrophilia (Ellis, 1977; Ellsaesser and Clem, 1986; Weyts et al., 1998a; Wojtaszek et al., 2002). Neutrophilia is also observed in inflammatory diseases associated with infectious agents (Ellsaesser et al., 1985; Weyts et al., 1998b). Weinreb (1958) observed thrombocytopaenia in rainbow trout (*Oncorhynchus mykiss*) as a result of stress, and injection with adrenocorticotrophic hormone (ACTH) and cortisone. Differential leukocyte counts in fish, as in mammals, may thus serve as an indicator of organism health. Total leukocyte counts (WBC) and differential leukocyte counts (DWBC) are typically performed manually, using a haemocytometer and differential staining of blood smears with Romanowsky stain, respectively (Hesser, 1960; Blaxhall, 1972; Campbell and Ellis, 2007b). Coulter counters have been demonstrated to be unsuitable for differentially counting fish leukocytes as the presence of the nucleus in erythrocytes renders them indistinguishable from leukocytes (Huffman and Arkoosh, 1997). Enrichment of leukocytes from fish blood may be performed by removing erythrocytes and thus facilitate differential counting. This may be achieved using density gradient centrifugation of blood (Waterstrat et al., 1988; Rowley, 1990) or hypotonic lysis of erythrocytes (Crippen et al., 2001; Inoue et al., 2002). Limitations in manual differential counting methods include the time-consuming nature of the procedure, morphological ambiguity between leukocytes (Ellis, 1977) and possible loss of cells when using lytic and density centrifugation procedures. Thus, the evaluation of whole blood may be the most appropriate strategy to determine accurate differential counts, provided that suitable tools are available.

Objective haematological evaluation of the physiological state of fish is thus dependent on not only understanding the individual morphologies, functions and responses of cells to intrinsic and extrinsic factors, but also the ability to accurately characterise, differentiate and enumerate blood cell types (Hrubec and Smith, 2000; Campbell and Ellis, 2007b). Flow cytometry (FC) is an instrumental technique that has achieved widespread use in human diagnostic haematology. The major advantage of FC technology is the ability to enumerate and differentiate several thousand cells per second. Flow cytometry has been applied

in the study of fish haematology, particularly in evaluating immune responses and phagocytic properties of leukocytes *in vitro* (Thuvander et al., 1987; Weyts et al., 1998a; Weyts et al., 1998b; Chilmonczyk and Monge, 1999; Scharsack et al., 2001). Development of total and differential leukocyte counting methods have been reported using cell light-scattering properties and combinations of scatter data and non-specific dye fluorescence (Morgan et al., 1993; Kfoury Jr et al., 1999; Inoue et al., 2002). These studies have generally found good correlations between FC and manual cell counts using whole blood and leukocyte fractions isolated by gradient separation or hypotonic lysis. However, a common problem reported was the inability to sufficiently resolve the thrombocytes and lymphocytes. Hübl *et al.* (1997) developed a five-part differential count on human leukocytes using fluorescent-labelled monoclonal antibodies (mAb) to each cell isotype. They reported good correlation with manual differential counts but erythrocyte lysis and washing steps appeared to have some influence on counts. However, the work did demonstrate the analytical scope of mAb techniques.

Shapiro (2003d) has stated: “To get a true ‘gold standard’ count, it would be advisable to work with unfixed, unlysed whole blood samples and ‘no-wash’ staining, using a vital nuclear stain to identify nucleated cells for triggering, to produce a differential count unbiased by any selective cell loss that might occur during lysis, fixation and/or washing. This seems entirely feasible”. 3,3'-Dihexyloxacarbocyanine iodide (DiOC₆(3)) is one of a group of fluorescent dyes which non-specifically stain mitochondria and endoplasmic reticulum (ER; Sabnis et al., 1997), and has been previously used to determine partial differential leukocyte counts in carp (*Cyprinus carpio*; Inoue et al., 2002). Hoeger *et al.* (2004) have utilised a fluorescent secondary-labelled mAb specific to rainbow trout thrombocytes to monitor cell activity in splenic tissue. The availability of DiOC₆(3), a dye already extensively evaluated by Shapiro (2003c) using FC on human blood, and the availability of a rainbow trout thrombocyte mAb jointly presented an opportunity to evaluate a combination of FC techniques in determining RBC, WBC and DWBC simultaneously on whole blood from fish without resorting to lysis or washing techniques.

The objectives of this study were to 1) develop procedures for the assessment of compatibility of anticoagulant and diluent formulations with fish blood, 2) evaluate the effectiveness of a fluorescent dye in resolving cell isotype populations at a range of concentrations using FC, 3) determine if dye compromised cell viability at dosages used to isolate cell types, negating its application in cell culture or live cell *in vitro* procedures, and 4) develop an integrated RBC, WBC and DWBC counting protocol. Rainbow trout were chosen as a model for this study because the physiology and haematology of this species has been evaluated extensively, and the teleosts are more closely related to other bony vertebrates, including man, in terms of similarities in blood cell morphology and function (Hrubec and Smith, 2000).

2.2 Materials and methods

2.2.1 Animals

Twenty yearling rainbow trout (*Oncorhynchus mykiss*) were obtained from the Eastern Region Fish and Game hatchery at Ngongotaha near Rotorua, New Zealand. Yearling trout were housed at Scion in indoor 500 L flow-through tanks (200 L h⁻¹), supplied with constant aeration and dechlorinated Rotorua city tap water at 12 ± 0.5°C. Approximately 3-year old adult rainbow trout already at Scion were also used in this study. Adult trout were housed in outdoor 12,000 L flow-through tanks (>1,000 L h⁻¹), supplied with constant aeration and dechlorinated tap water. Fish were fed ad libitum every second or third day with a commercial salmon feed (Reliance Stockfoods, Dunedin, NZ).

2.2.2 Sampling protocol

Fish were anaesthetised (Stage 3 anaesthesia; Hrubec and Smith, 2000) using 0.1 g L⁻¹ tricaine methane sulphonate (MS222; Sigma Aldrich, Australia) in tank water neutralised to pH 7 with sodium bicarbonate (Campbell and Ellis, 2000b). Blood samples were collected by caudal venipuncture using 1 mL syringes fitted

with 0.5 x 25 mm (25 gauge) needles (Terumo, USA) pre-dosed with heparin or EDTA depending on the experiment.

2.2.3 Traditional haematology

A variety of analytical techniques were employed during this study. Traditional haematological techniques, including Hct and Hb, were used to assess the effects of anticoagulant formulations and diluent solutions on blood parameters. Manual RBC, WBC and DWC were performed to compare with automated (FC) counting techniques. Cytochemical evaluation of blood smears was performed to identify properties of blood cells that could be used to characterise morphologically ambiguous cell types.

2.2.3.1 Haematocrit

Haematocrit, measured as the percentage packed red cell volume, was determined using the micro-haematocrit method (Bain and Bates, 2001). Anticoagulated whole blood was drawn into 5 μ L micropipettes (Drummond Scientific Company, USA), sealed with Crit-O-Seal (McCormick Scientific, UK) and centrifuged for 5 min at 15,000 rpm in an EBA 21 centrifuge (Hettich, USA) fitted with a microhaematocrit rotor.

2.2.3.2 Haemoglobin

Haemoglobin in whole blood, diluted blood and blood plasma was determined using the cyanmethaemoglobin (HiCN) method as described by Bain and Bates (2001). A modified Drabkin's solution was prepared, as the standard Drabkin's solution (Bain and Bates, 2001) is known to cause interference with plasma proteins in non-mammalian species (N. Ling, Waikato University, personal communication). The modified reagent was made using 200 mg $K_3Fe(CN)_6$ and 50 mg KCN made up to 1 L with milliQ water and adjusted to pH 9.6. Four

microlitres of whole blood was added to 1 mL of modified Drabkin's solution; either 100 μL of whole blood plasma or 200 μL of diluted blood supernatant, prepared as in Section 2.2.5.1, was added to 900 μL or 800 μL of modified Drabkin's solution, respectively. These solutions were thoroughly mixed and absorbance at 540 nm measured using a Metertek SP-830 UV-visible spectrophotometer. Haemoglobin concentration was calculated relative to a HiCN standard (Sigma, Australia), as described by Bain and Bates (2001).

2.2.3.3 Total red blood cell count

Total red blood cell counts were performed using an improved Neubauer haemocytometer (Hawksley, England) as described by Dacie and Lewis (1991). Two microlitres of whole blood were fixed in 0.5 mL of red blood cell diluting fluid. A sample of the fixed cell suspension was introduced into the haemocytometer, which was examined at 100 x magnification with a Zeiss Axioplan 2 light microscope. Images were captured with an AxioCam HRC camera and analysed using ImagePro Plus[®] software (Media Cybernetics Inc., Silver Springs, MD). The total number of cells in an area encompassing 14.91 large squares (0.44 mm^2) of the haemocytometer was determined using the software particle counting function. Total red blood cell count was then determined from the volume represented by the area counted ($0.004 \mu\text{L square}^{-1}$) and the blood dilution factor according to the formula:

$$\text{cells L}^{-1} = \frac{\text{total cell count} \times 251 \text{ (blood diln.)} \times 1 \times 10^6 \times 1.1 \text{ (anticoagulant diln.)}}{14.91 \times 0.004}$$

2.2.3.4 Total leukocyte count

Total leukocyte counts were performed with an improved Neubauer haemocytometer using a modification of the method described by Bain and Bates (2001) for analysis of human blood samples. As an erythrocyte lysis procedure could not be performed on fish blood, whole blood was diluted according to the method described by Shaw (1930). Shaw's Solution A was prepared by dissolving 25 mg Neutral Red (BDH, UK) and 0.9 g sodium chloride (JT Baker; Biolab, NZ)

in 100 mL distilled water. Solution B contained 12 mg Crystal Violet (BDH, UK), 3.8 g sodium citrate (Sigma, NZ) and 0.4 mL 37% formaldehyde (Merck, NZ) dissolved in 100 mL distilled water. Two microlitres of whole blood was diluted in 50 μ L of diluent, comprised of 25 μ L each of solutions A and B, in a 1 mL centrifuge tube (Eppendorf, Germany) and mixed. A sample of the cell suspension was introduced into the haemocytometer and the total number of leukocytes counted in the four large corner squares of the haemocytometer, representing 0.4 μ L of the total sample delivered. Total leukocyte count was then calculated using the blood dilution factor according to the formula:

$$\text{cells L}^{-1} = \frac{\text{leukocte count} \times 26 \text{ (blood diln.)} \times 1 \times 10^6 \times 1.1 \text{ (anticoagulant diln.)}}{0.4}$$

2.2.3.5 Differential leukocyte count

Differential leukocyte counts were performed on blood smears prepared with 2 μ L anticoagulated whole blood on LabServ[®] Polysine[®]-coated microscope slides (Biolab, New Zealand). Smears were air-dried for at least 30 min and fixed for 5 min in HPLC-grade methanol (Merck, Germany). Human blood controls were prepared in the same way using freshly-sampled blood, without anticoagulant, obtained using a lancet (BD, USA). Fixed blood smears were then stained with Leishman's-Giemsa (Kent et al., 2007), May-Grunwald-Giemsa (Bain and Lewis, 2001) and Wright-Giemsa (Campbell and Ellis, 2007a) stains. Stained smears were air-dried overnight and coverslipped using Clarion[™] mounting medium (Sigma, Australia).

For each slide, an examination of a complete head-to-tail subsection of the smear was performed. Leukocytes were differentiated based on morphology and metachromatic staining of cytoplasm, cytoplasmic granules and nuclear chromatin (Hrubec and Smith, 2000; Campbell and Ellis, 2007) and the relative proportions of each cell type expressed as a percentage of the total leukocytes counted. The performance of each stain was evaluated in terms of intensity of staining and the relative ability to differentiate the leukocyte types. Smears of blood samples treated with heparin and EDTA were similarly compared.

2.2.3.6 Cytochemistry

Cell cytochemistry was examined to assist with the resolution of remaining leukocyte ambiguity in Romanowsky-stained blood preparation. Cytochemical staining of whole blood smears (Section 2.2.3.5) and leukocyte populations isolated by flow cytometry (Section 2.2.4) was performed according to the methods of Tavares-Dias (2006a; 2006b). Tests for sudanophilia (positive staining using Sudan Black B, indicating presence of lipids such as phospholipids, neutral fats and sterols), Periodic acid-Schiff (PAS)-positivity (indicating mucopolysaccharides), myeloperoxidase, alkaline phosphatase and non-specific esterase in leukocytes were performed using commercial diagnostic kits (Sigma, Australia). Fixing and staining of samples was performed as prescribed for each kit. Stained and dried smears were coverslipped using Clarion™ or aqueous-based Gel/Mount™ (Biomedica, USA) mounting media, as recommended.

In addition to commercial kits, blood smears were also stained with 0.5% Toluidine Blue (TB; BDH, UK) at pH 9.0 after fixation for 5 min in methanol, and with 0.2% Toluidine Blue at pH 3.2 after fixation with 1% lead subacetate (bis(acetate)tetrahydroxytrilead; BDH, UK) in 70% ethanol (Merck, Germany). These tests have previously been used to positively identify the thrombocytes and basophils, respectively (Tavares-Dias, 2006a; 2006b). Identification of reticulocytes (immature red blood cells) was performed by staining vital cells with Brilliant Cresyl Blue (BCB; BDH, UK). Whole blood and 1% BCB solution in 0.65% sodium chloride (Merck, Germany) were mixed 1:1 v/v and incubated at 37°C for 20 min (Tavares-Dias, 2006b). A similar mixture was prepared and incubated at 22°C to determine any adverse thermal effects on cellular integrity at the higher incubation temperature. After incubation, blood smears were prepared from these mixtures and dried, fixed and stained with Leishman's-Giemsa stain as already described in Section 2.2.3.5.

2.2.3.7 Photomicroscopy

Differential leukocyte counts and all other aspects of morphological photomicroscopy in this study were performed using an Olympus® BS61

microscope equipped with a ColorView[®] III digital camera, at 1,000 x magnification under oil immersion as required. Images were viewed using AnalySIS LS software (Olympus, USA).

2.2.4 Flow cytometry

Blood samples subjected to various conditions, as described in Section 2.2.5, were analysed by FC. Methodology was adapted from established protocols for human blood FC analysis, but modified as required due to the nucleated nature of fish erythrocytes. This section describes the instrument and generalised operational conditions and protocol.

2.2.4.1 Flow cytometer

Flow cytometric analysis was performed on a FACSVantage[™] SE flow cytometer (BD Biosciences, USA), equipped with the BD FACSDiVa[™] digital data processing electronics and software option. The FC was fitted with a Coherent INNOVA[®] Enterprise[™] II ion laser regulated at 300 mW, providing 488 nm excitation, and a Spectra-Physics Model 127 helium neon laser, providing 633 nm excitation. The instrument sheath fluid was phosphate-buffered saline (PBS; Gibco, USA), adjusted to pH 7.2 and delivered through a 70 µm nozzle at 131 kPa.

2.2.4.2 Tube preparation

Blood sample evaluations by FC were performed in surface-deactivated polypropylene tubes to minimise cell-tube adhesion and associated quantitation error. Five millilitre 12 x 75 mm polypropylene Falcon tubes (BD, USA) were filled with a 4% solution of bovine serum albumin (BSA; Sigma, Australia) in PBS and stored overnight at 4°C (Phi-Wilson and Recktenwald, 1994). Prior to use, the tubes were emptied and centrifuged at 3000 rpm for 5 min. The remaining

BSA solution was removed with a Pasteur pipette. Blood samples were generally diluted to $\approx 1 \times 10^6$ cells mL⁻¹, the optimal cell concentration in flow cytometry practice according to Shapiro (2003c), in PBS prior to fluorescent dye staining. This was typically achieved by adding 4 μ L of whole blood to 4 mL of diluent. TruCount™ fluorescent beads (BD, USA) were included with samples to facilitate quantitation. TruCount™ tubes contain an accurately known pre-dosed number of fluorescent beads ($\approx 50,000$ beads per tube). One millilitre of PBS was added to a TruCount™ tube and thoroughly mixed on a vortex mixer. A 100 μ L suspension ($\approx 5,000$ beads) was then added to each tube prior to the final staining step described below.

2.2.4.3 Fluorescent dye staining

Diluted blood samples were stained with 50 to 1,000 μ M concentrations of 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3); Molecular Probes, USA). A 1 mM stock solution of DiOC₆(3) in dimethylsulphoxide (DMSO; Sigma, Australia) was diluted with DMSO to prepare 10 and 100 μ M working solutions, and kept in the dark at room temperature. Stock solutions were added to cell suspensions as required to achieve the final concentration of dye. The total combined DMSO and dye solution volume was kept constant (20 μ L). Generally, DMSO concentration should not exceed 1% of the sample by volume to minimise cytotoxic effects (Shapiro, 2003c). Samples were incubated for 15 min in the dark at room temperature. Protein additives such as BSA were not included in preparations due to potential interference with the lipophilic DiOC₆(3) dye (Shapiro, 2003c). Non-viable cells were stained with Sytox[®] Red Dead Cell Stain (Molecular Probes, USA). Two microlitres of Sytox[®] Red was added to working cell suspensions and incubated for 2 min in the dark at room temperature as the last step (i.e. after the addition of fluorescent beads) prior to FC analysis.

2.2.4.4 Monoclonal antibody and fluorescent conjugate labelling

In assessments using mAb to specifically identify the thrombocytes, 5 μL of mAb stock solution was incubated with 1 mL cell suspensions of $\approx 5 \times 10^6$ cells mL^{-1} at 4°C for 20 min. One hundred microlitre TruCount™ bead suspensions were added as an internal standard and assumed to account for cell losses during washing and reconstitution steps. The sample was centrifuged at 2000 rpm for 10 min at 4°C and the pellet resuspended in 4 mL PBS. The centrifugation was repeated and the pellet re-suspended in 1 mL PBS. The sample was then incubated with 5 μL of fluorescent conjugate mAb stock solution for 20 min at 4°C . The suspension was centrifuged and resuspended in 4 mL PBS. Fluorescent dye was then added as required and incubation performed as described in Section 2.4.3 prior to FC analysis.

2.2.4.5 Instrument parameters

For all sample analyses, the event threshold was set on the forward scatter (FSC) channel at a value of 1000. Thresholding on fluorescence, as described by Shapiro (2003a) for analysis of human nucleated cells (leukocytes) without lysis of non-nucleated erythrocytes, was not possible due to the presence of nucleated erythrocytes in fish. The threshold was set as low as possible to enable identification of compromised cells using the Sytox® Red stain, which could then subsequently be excluded by gating. A low threshold is also important during cell sorting, as events falling below the threshold can not be excluded using Boolean gating strategy and may be included with gated events, resulting in significant contamination of sorted populations with low cell counts.

Fluorescence was measured on multiple channels. 3,3'-dihexyloxycarbocyanine iodide fluorescence, corresponding with fluorescein isothiocyanate (FITC) fluorescence at 500-550 nm, was measured on the FC FL1 channel, equipped with a 530/30 nm bandpass filter. Sytox® Red stain fluorescence, corresponding with allophycocyanin (APC) fluorescence at 640-680 nm, was measured on the FC FL6 channel, equipped with a 660/20 nm bandpass filter. APC-positive DiOC₆(3)-stained events could then be discriminated on SSC

vs. FL1 dotplots by highlighting these in a different colour using the DiVa software. The FSC and side scatter (SSC) channel photomultiplier detector voltages were set to 390 and 320 mV, respectively. The FITC, APC and phycoerythrin (PE) channel voltages were set to 350, 500 and 430 mV, respectively. The PE channel, together with the FITC channel, was used for isolation of TruCount™ bead fluorescence from labelled-cell fluorescence.

2.2.4.6 Cell counting

Single-parameter histograms and dual-parameter dot plots of the various channel events were monitored and used for setting gates. The total number of measured events per FC analysis was pre-determined by operator ability to visually resolve populations and thus enable manual construction of gates around populations of interest. The total number of measured events, ranging from $\approx 30,000$ to $\approx 135,000$, was regulated by counting a fixed number of TruCount™ beads per experimental sample set, typically between 30 and 100. The total measured event per second rate was maintained at 1,000. The number of cells for each identified population was calculated using the following formula:

$$\text{cells L}^{-1} = \frac{\text{cell count} \times \text{measured bead count} \times 1 \times 10^6 \times 1.1 \text{ (anticoagulant diln.)}}{\text{actual bead count} \times \text{blood volume } (\mu\text{L})}$$

2.2.4.7 Cell sorting

For fluorescence-activated cell sorting (FACS), the 4-way sorting instrument function was enabled. Optimal sorting was achieved using a drop drive frequency of 39 kHz and an amplitude setting of 3 V. Drop delay was typically maintained at a setting of ≈ 14.5 . Sorting was performed using the Purity sorting option on the DiVa software to maximise purity of sorted cell populations, but at the expense of yield of rare cells. Cells were sorted into coated FC tubes containing 4 mL 2% BSA in PBS, maintained at $\approx 4^\circ\text{C}$. Immediately after collection, tubes were

centrifuged at 2,000 rpm at 4°C for 10 min and the cells re-suspended in \approx 250 μ L remaining supernatant for cyto centrifugation onto slides.

Cyto centrifugation was performed using a Shandon Cytospin[®] 4 cyto centrifuge (Thermo Electron Corporation, USA). Shandon EZ Cytofunnels[®] were coated with BSA to minimise cell losses using a similar procedure as for FC tubes (Section 2.4.2). Cytofunnels were rinsed with distilled water to remove contaminant fibres originating from the attached absorbent filter cards, filled with 650 μ L 4% BSA on an inclined surface to prevent overflow and kept overnight at 4°C. The BSA solution was discarded prior to use, after which funnels were fitted with plain glass slides and centrifuged at 2,000 rpm in the cyto centrifuge for 5 minutes to remove any remaining BSA solution. The funnels were then fitted with coated Shandon Cytoslides[®] with outlined specimen areas (Thermo Electron Corporation, USA) and 50 μ L PBS added. The funnels were centrifuged at 2000 rpm for 1 min to wet the attached absorbent filter cards, which decreased the rate of absorption of diluent from cell suspensions and reduced loss of cells from the slide surface. Cell suspensions, no greater than 250 μ L in volume, were centrifuged at 750 rpm for 10 min and the cell isolates air-dried for 30 min before fixing and staining as per Section 2.3.

2.2.5 Experimental protocol

The following section outlines the development of a rapid and accurate methodology for: 1) total and differential analysis of peripheral blood of rainbow trout, including selection of effective and compatible anticoagulant and diluent formulations, 2) optimisation, benefits and limitations of the use of non-specific fluorescent dyes and monoclonal antibodies and 3) a comparison of instrumental (FC) and traditional manual techniques.

2.2.5.1 Anticoagulant evaluation

Two anticoagulant stock solutions were prepared in PBS; 5,000 i.u. mL⁻¹ ammonium heparin (Sigma, Australia) and 40 mg mL⁻¹ di-sodium EDTA (Sigma,

Australia) neutralised with NaOH. Syringes were pre-dosed with anticoagulant solution using a micropipette. Nine hundred microlitre blood samples were collected from adult trout ($n = 10$) using syringes containing 100 μL of anticoagulant solution. Final concentrations of heparin and EDTA in blood prepared in this way were 500 i.u. mL^{-1} and 4 mg mL^{-1} , respectively. These have been determined to be optimal dosages for fish blood in terms of prevention of coagulation and preservation of leukocyte viability (Mainwaring and Rowley, 1985). Needles were removed and blood samples transferred into 1.5 mL centrifuge tubes, mixed thoroughly and stored on ice. The effects of the two anticoagulants were evaluated by measuring Hct, whole blood Hb and plasma Hb concentrations as described in Section 2.3 immediately (T0) and again after 2 h on ice (T120). The osmolality (mOs kg^{-1}) of plasma samples was measured on an Advanced Osmometer 3 D3 (Norwood, USA). The osmolality of PBS and aqueous solutions of heparin and neutralised EDTA were also measured to calculate the individual osmolalities of the components of the anticoagulant solutions.

2.2.5.2 Diluent compatibility evaluation

The compatibility of PBS and IsoTon[®] II (Beckman Coulter, USA) as fish blood diluents and FC sheath fluids was evaluated. Based on the results of the anticoagulant evaluation, heparin was selected as the anticoagulant of choice. Blood samples were collected from adult trout ($n = 10$) as previously described. Blood samples were diluted 4 times by volume in PBS and IsoTon[®] II, both containing 500 i.u. mL^{-1} ammonium heparin to maintain anticoagulant concentration, and kept on ice for 2 h. The effect of dilution on erythrocyte lysis was evaluated by comparing Hb concentrations in diluted blood supernatant and whole blood plasma, after centrifugation for 5 min at 6,000 rpm, as described in Section 2.3

2.2.5.3 Fluorescent staining evaluation

In order to determine the possible effects of DiOC₆(3) on blood cells and to establish optimal dosing for identification and enumeration of blood cells, blood samples were subjected to a geometric concentration range (0, 25, 50, 100, 250, 500 and 1,000 nM) of DiOC₆(3) in DMSO. The final total volume of DiOC₆(3) and DMSO was kept constant (20 µL). Approximately 500 µL blood samples in heparin were collected from yearling trout (n = 10) as previously described. Blood samples were first analysed by FC in the absence of fluorescent staining to establish approximate total blood cell counts. This was achieved using 4 µL of blood diluted in 4 mL of PBS in the presence of counting beads. Based on the preliminary cell counts, between 2.6 and 4 µL blood samples were used to obtain the recommended 1×10^6 cell mL⁻¹ for FC enumeration during the staining exercise. Blood samples diluted in this way were then treated with DiOC₆(3), 100 µL of the counting bead suspension added and incubated at room temperature. After 18 min, 2 µL of Sytox[®] Red was added, samples gently mixed and then incubated for a further 2 min. After approximately 20 min, samples were analysed by FC. To obtain accurate total red cell and leukocyte counts, 100 TruCount™ beads were counted for each sample. Gates were drawn within the DiVa software around populations resolved on fluorescence parameter histograms and dot plots of SSC vs. FL1 fluorescence, and quantitative analysis performed based on bead and event counts. Fluorescence-activated cell sorting (FACS) was performed on the resolved populations of 5 specimens using 50 nM DiOC₆(3) (Shapiro, 2003c). Cell isolates were prepared on cytocentrifuge slides, fixed and treated with Leishman's-Giemsa stain or cytochemical stains as previously described to characterise the blood cell types.

2.2.5.4 Flow cytometric count validations with fluorescent dye only

Total and differential FC cell counts, based on the characterisation of trout blood cells using the optimal DiOC₆(3) dose (50 nM) were compared with haematological data obtained using manual methods (Section 2.2.3). Method

comparisons were performed using blood samples collected from yearling rainbow trout (n = 10).

2.2.5.5 Flow cytometric count validations with fluorescent dye and thrombocyte antibody

Thrombocytes were discriminated from other leukocytes using anti-trout mAb 42, generously donated by Dr. Bernd Köllner (Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Germany). Using an APC-labelled secondary mAb, the number and distribution of thrombocytes could be determined on dual parameter (SSC vs FL1) dot plots of cells non-specifically stained with DiOC₆(3) after mAb labelling. APC-fluorescent events (thrombocytes) were highlighted on dot plots in the same manner as Sytox[®] Red-positive events as described. Blood samples from the specimens used in 2.5.4 above were prepared for FC analysis with mAb 42. Secondary antibody labelling was performed with anti-mouse IgG1 mAb X56 APC (BD, USA). Samples were then incubated with 50 µM DiOC₆(3) for 20 min and analysed on the FC.

2.2.6 Statistical analyses

Statistical comparison of anticoagulant effects on Hct and plasma Hb was performed by two-way analysis of variance (ANOVA) with time and anticoagulant as factors, followed by a Tukey's HSD post-hoc test where significant factor effects were observed. Effects of anticoagulants on erythrocyte dimensions (cell length and width) were independently analysed by Paired T-tests. For comparison of effects of diluents on plasma Hb, a Wilcoxon Matched Pairs Test was instead performed as data did not conform to the assumptions of parametric analysis following log-transformation. To assess the cytotoxic effects of DiOC₆(3), proportional cell viability data were first arcsine transformed (Sokal and Rohlf, 1973). These data were then analysed using the non-parametric Kruskal-Wallis ANOVA due to departure from normality. For comparative total RBC counts using manual and DiOC₆(3) methods, and total WBC counts using

manual and mAb-DiOC₆(3) methods, paired T-tests were performed. For comparison of total RBC data using the three counting methods (manual, DiOC₆(3) and mAb+DiOC₆(3)), a one-way ANOVA was performed on log-transformed data followed by a Tukey's HSD post-hoc test where a significant method effect was observed. To compare DWBC data for the three counting methods, proportional data were arcsine transformed and analysed using a Wilcoxon matched pairs test. All statistical analyses were performed using STATISTICA v8.0 software (Statsoft, Tulsa, OK, USA). The critical level of statistical significance for all tests was $\alpha = 0.05$.

2.3 Results

2.3.1 Anticoagulant evaluation

Significant differences were observed in the effects of heparin and EDTA on trout blood parameters, as summarised in Fig. 2.1. There was a significant anticoagulant effect ($P < 0.05$, ANOVA) on mean haematocrit values. However, significant differences ($P < 0.1$, Tukey's HSD post-hoc test) were only observed between values at T0. A significantly higher plasma haemoglobin level was observed in the EDTA-anticoagulated blood at 120 min. Mean plasma osmolalities (\pm SEM) after 120 min were 316 ± 3.96 mOs kg^{-1} for heparin-treated blood and 406 ± 1.73 mOs kg^{-1} for EDTA-treated blood. The osmolality of aqueous heparin (5000 i.u. mL^{-1}) was 47 mOs kg^{-1} and that of aqueous EDTA (40 mg mL^{-1}) was 904 mOs kg^{-1} .

Photomicroscopic differences in background staining effects were observed between blood smears treated with heparin and EDTA, and differences in intensity of staining were observed between the three Romanowsky stain formulations. A comparison of smears prepared from heparin and EDTA-treated blood ($n = 5$ for each), stained individually with Leishman's-Giemsa (LG), May-Grunwald-Giemsa (MGG) and Wright's-Giemsa (WG) formulations, is presented in Plate 2.1. Basophilic staining was generally more intense in EDTA-treated samples. In LG- and MGG-stained smears of heparinised blood, cells were surrounded by a faint, pink haze. This was not evident in EDTA-treated blood stained with LG and MGG, or in WG-stained blood treated with either anticoagulant. Generally, cell size appeared to be reduced in EDTA-treated blood smears. Mean cell length and width ($n = 50$) of the predominant cell type (erythrocytes) as measured in smears is presented in Fig. 2.2. Erythrocyte length and width were significantly lower ($P < 0.05$, paired T-test) in EDTA-treated blood. Staining of erythrocyte cytoplasm was faintly eosinophilic and was comparable for both anticoagulants and all staining procedures. In both heparin and EDTA-treated blood, LG and MGG were comparable in terms of intensity of basophilic staining of cell nuclei and leukocyte cytoplasm compared with WG, which exhibited less intense staining.

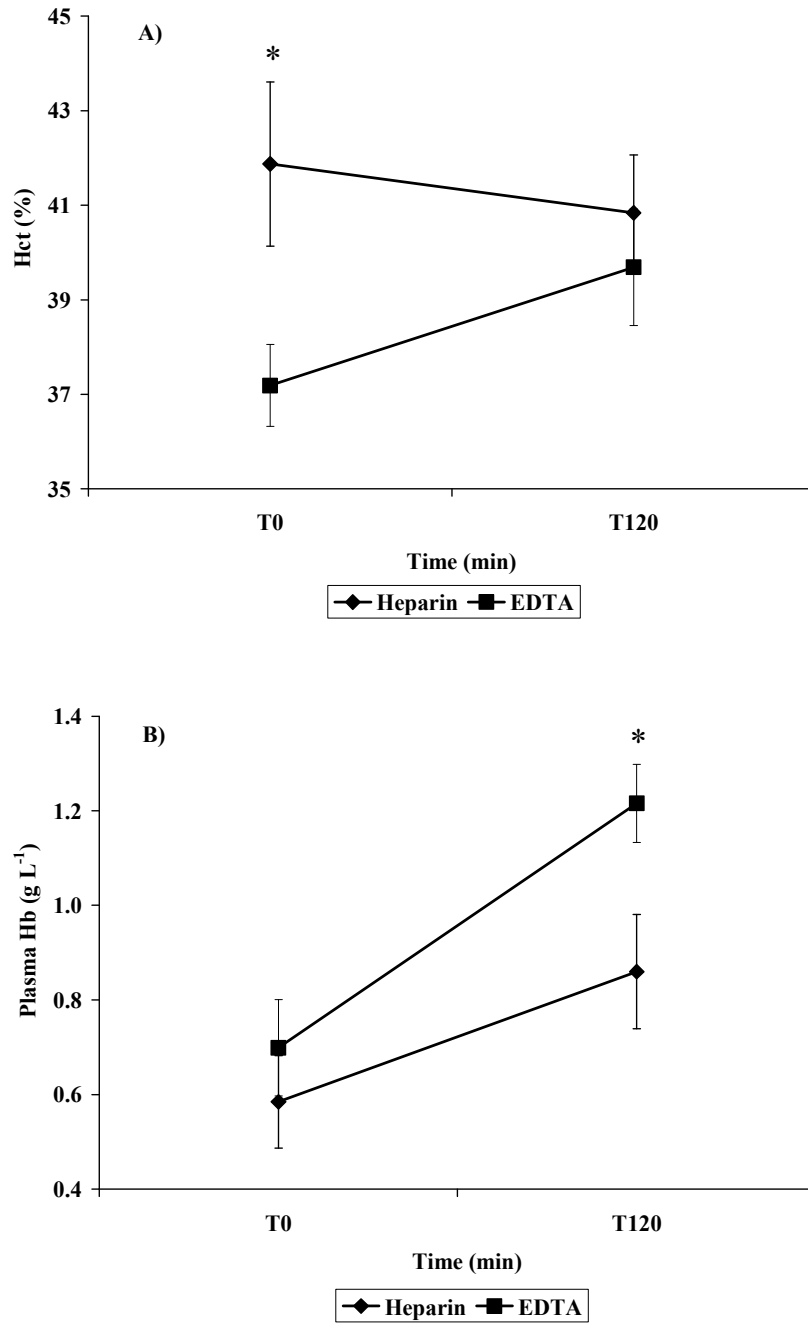


Figure 2.1. Blood parameters (haematocrit (A) and plasma haemoglobin (B)) of rainbow trout blood ($n = 10$) incubated with 500 i.u. mL^{-1} heparin and 4 mg mL^{-1} EDTA for 120 min. Asterisks indicate significant differences ($P < 0.1$, Tukey's HSD test). Values are mean \pm SEM.

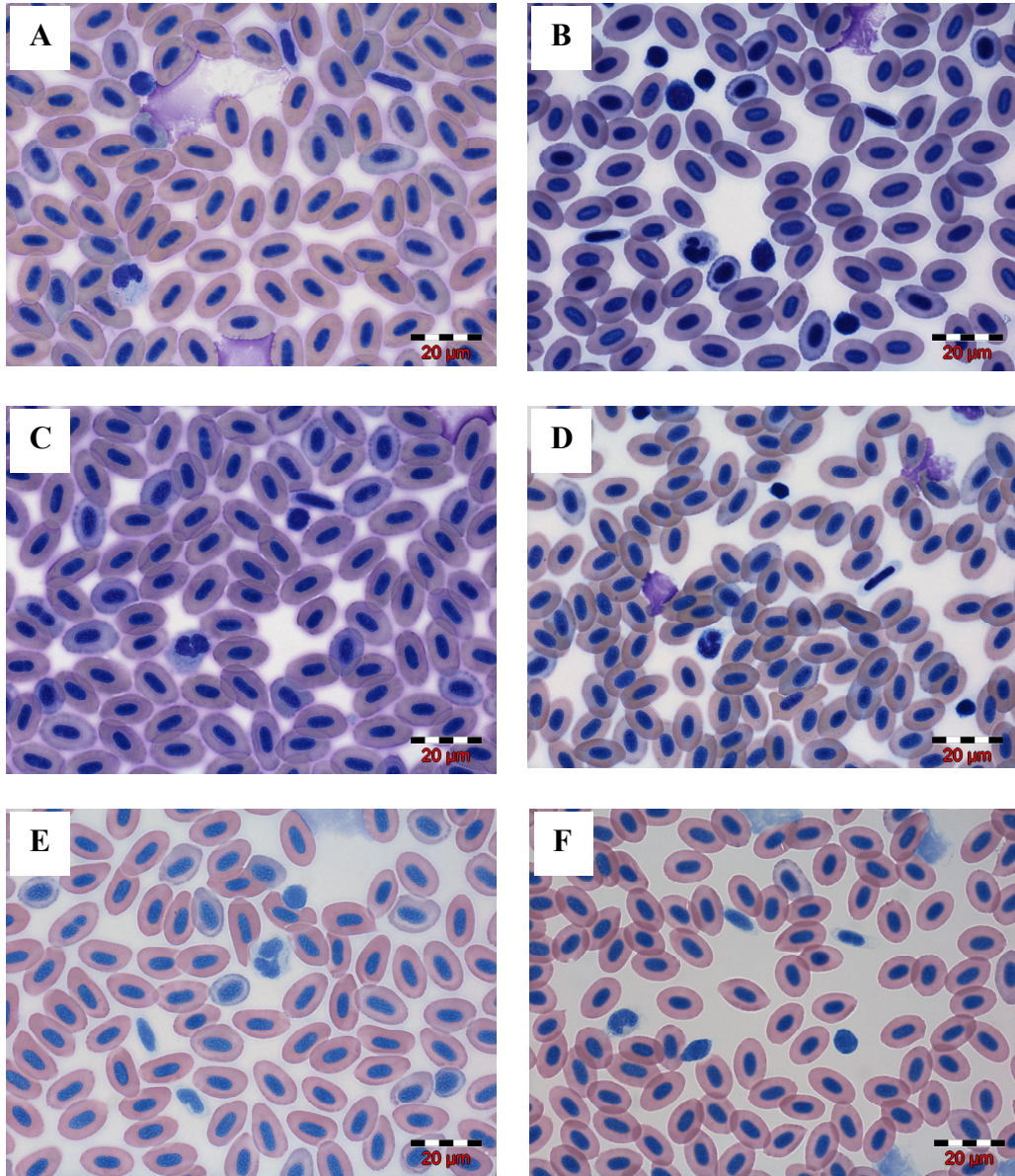


Plate 2.1. Staining characteristics of blood cells sampled in heparin (A, C & E) and EDTA (B, D & F) and stained with Leishman's-Giemsa (A & B), May-Grünwald-Giemsa (C & D) and Wright's-Giemsa (E & F) formulations.

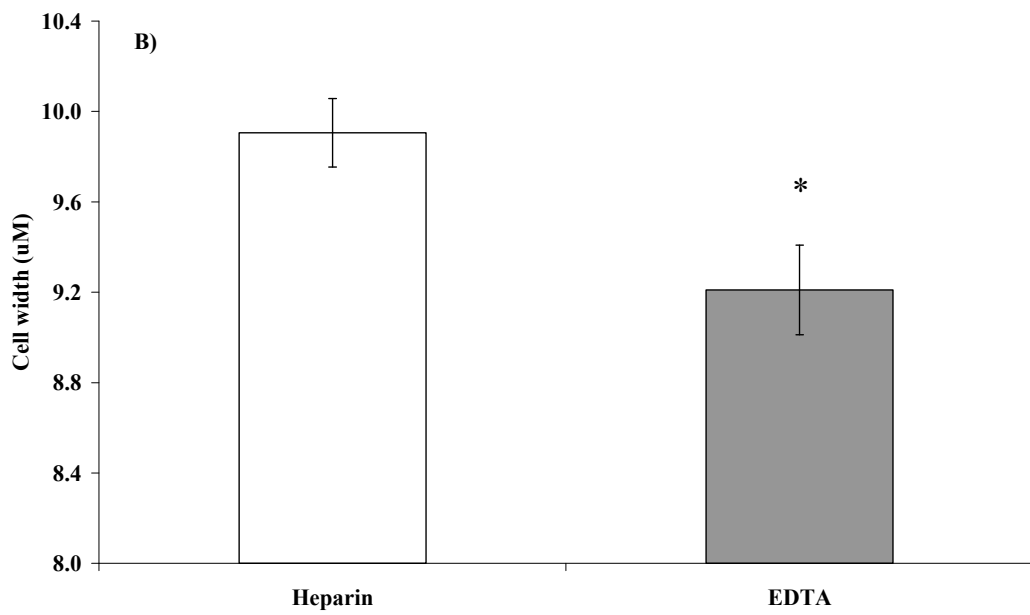
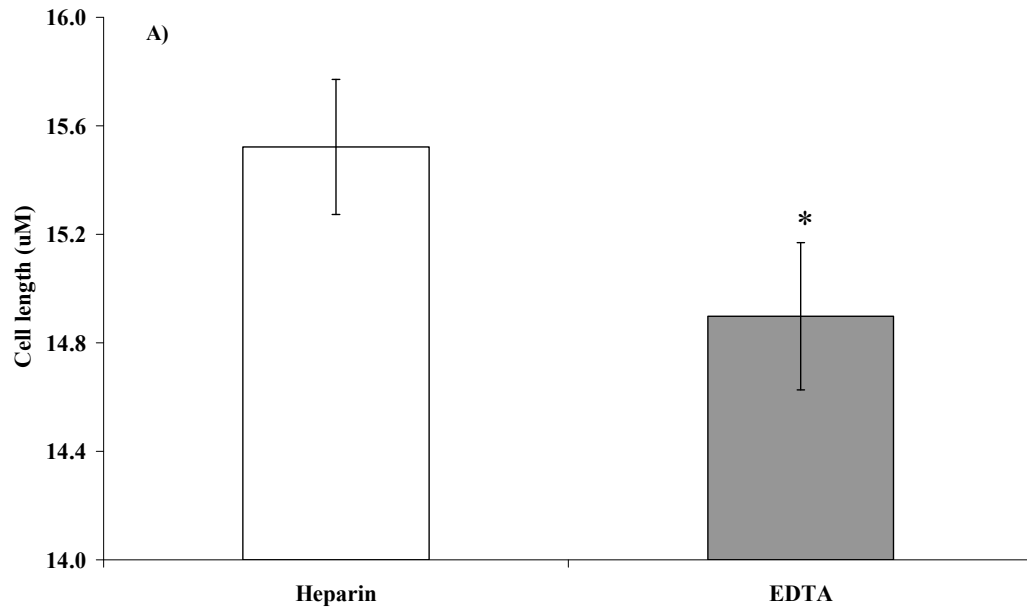


Figure 2.2. Mean ($n = 50$) erythrocyte cell length (A) and width (B) for blood treated with 500 i.u. mL^{-1} ammonium heparin and 4 mg mL^{-1} neutralised EDTA. Asterisks indicate significant differences ($P < 0.05$, paired T - test). Values are mean \pm SEM.

2.3.2 Diluent compatibility evaluation

A significant increase ($P < 0.05$, Wilcoxon matched pairs test) in centrifuged blood supernatant Hb was observed at T120 in blood diluted with IsoTon[®] II (Table 2.1). Mean (\pm SEM) undiluted plasma osmolality was 317 (1.23) mOs kg⁻¹. PBS and IsoTon[®]II solution osmolalities were 311 and 340 mOs kg⁻¹, respectively.

Table 2.1. Mean (\pm SEM) blood parameters of samples diluted 1:3 with PBS and IsoTon[®] II ($n = 10$ for each) and incubated on ice for 120 min. Asterisks indicate significant ($P < 0.05$, Wilcoxon matched pairs test) measured parameter differences between diluents.

Parameter	PBS	IsoTon II
Total haemoglobin (g L ⁻¹)	85.03 (3.93)	89.23 (3.58)
Diluted blood supernatant haemoglobin (g L ⁻¹)	0.51 (0.05)	2.36 (0.54)*
Erythrocyte haemolysis (%)	0.61 (0.06)	2.65 (0.55)

2.3.3 Characterisation of blood cell types and cytochemical staining

Cell dimensions and response to the various cytochemical and metachromatic stains is summarised in Table 2.2. Light microscopic evaluation results are presented in Plate 2.2.

2.3.3.1 Erythrocytes

Mature erythrocytes were elliptical in shape and characterised by faintly eosinophilic cytoplasm and deep blue, elliptical nuclei. Immature erythrocytes (reticulocytes) were less elliptical in shape with faintly basophilic cytoplasm. Nuclei were less elliptical and proportionately larger compared with mature cells. Staining with BCB at 37°C (Tavares-Dias, 2006b) to reveal reticulocytes resulted in extensive cell damage. Cell integrity was preserved after incubation at 22°C with BCB, resulting in basophilic staining of immature erythrocyte ribonucleoprotein, consistent with results reported by Tavares-Dias (2006b).

Table 2.2. Dimensions and cytochemical staining reactions of characterised rainbow trout blood cell types. - = negative, + = mildly positive, ++ = moderately positive, +++ = very positive, no result = not tested.

Cell isotype	Mean (\pm SEM) cell dimensions (n = 50)		Cytochemical staining reactions							
	Length (μ m)	Width (μ m)	Sudan Black B	Periodic acid-Schiff	Myelo-peroxidase	Nonspecific esterase	Alkaline phosphatase	Brilliant Cresyl Blue	Toluidine Blue pH = 3	Toluidine Blue pH = 9
Erythrocytes	15.5 (0.12)	9.9 (0.06)	-	-	-	-	-	-	-	-
Immature erythrocytes	13.0 (0.20)	9.9 (0.12)	-	-	-	-	-	+++	-	-
Lymphocytes	8.8 (0.19)	8.8 (0.19)	-	-	-	-	-	-	-	Purple-blue nucleus, blue cytoplasm
Granulocytes	13.4 (0.14)	13.4 (0.14)	+ to +++	- to +++	++ to +++	-	- to +++	-	-	-
Thrombocytes	12.0 (0.29)	6.2 (0.13)	-	-	-	-	-	-	-	Purple-blue nucleus, colourless cytoplasm

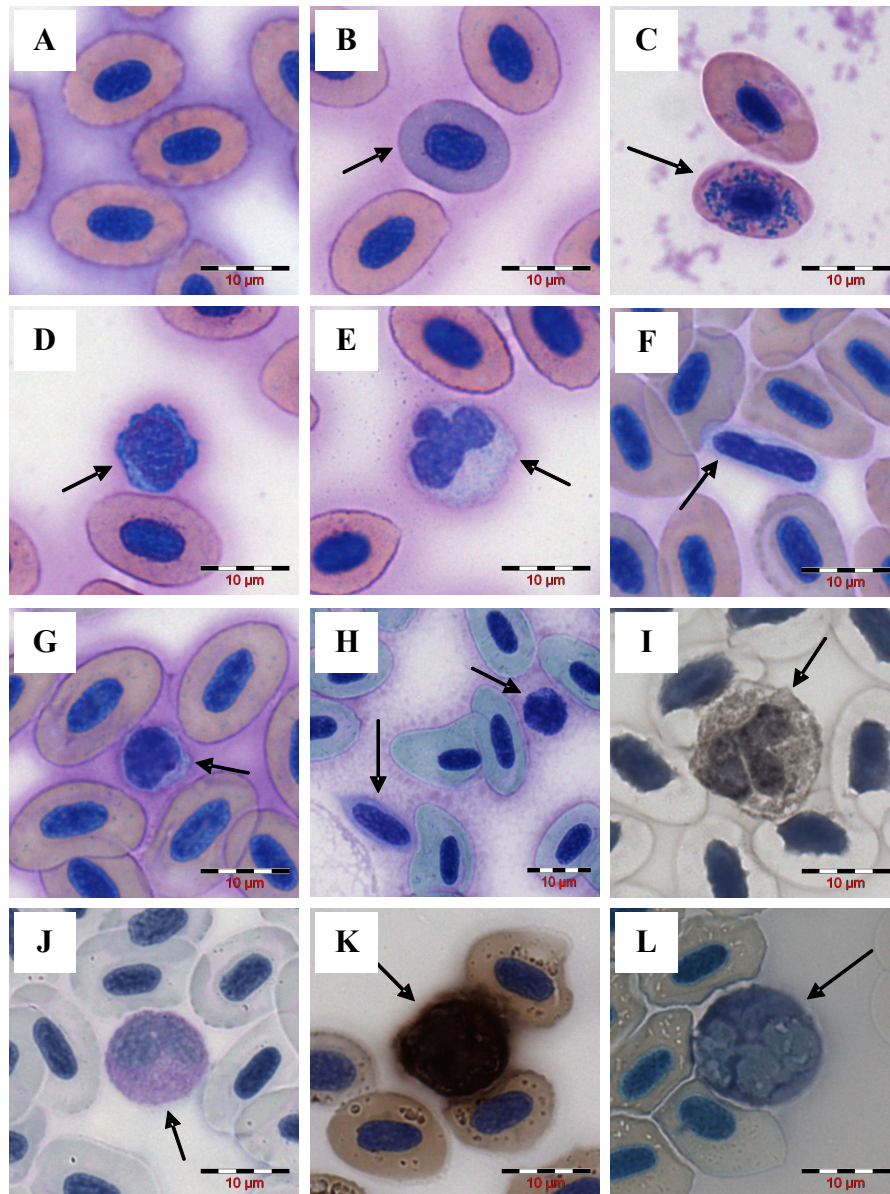


Plate 2.2. Characterisation of rainbow trout blood cell types (arrowed). Staining with Leishman's-Giemsa revealed erythrocytes (A), immature erythrocytes (B), lymphocytes (D), granulocytes (E) and spindle-shaped and ovoid to round thrombocytes (F,G). Brilliant Cresyl Blue staining confirmed the presence of immature erythrocyte ribonucleoprotein (C). Cytochemical staining revealed positive response of granulocytes to Sudan Black B (I), Periodic acid-Schiff (J), myeloperoxidase (K) and alkaline phosphatase (L) tests. Staining with Toluidine Blue at pH 9 (H) resulted in leukocyte staining comparable to LG.

2.3.3.2 Granulocytes

Granulocytes were round in shape, with eccentric nuclei which stained deep purple. The cytoplasm was generally pale blue-grey and granular, and did not respond to Romanowsky staining. Granulocytes with eosinophilic and basophilic granules were not observed. The granulocytes generally tested positive for sudanophilia, PAS activity, myeloperoxidase and alkaline phosphatase.

2.3.3.3 Lymphocytes

Lymphocytes varied in size, were typically round in shape and characterised by purple nuclei which occupied most of the cytoplasmic volume. Larger lymphocytes contained light purple-staining nuclear chromatin and occasionally presented lobed nuclei. The cytoplasm stained dark blue, which was the most characteristic feature of these cells when using Romanowsky staining for performing differential counts.

2.3.3.4 Thrombocytes

Thrombocytes exhibited a range of morphologies, typically spindle-shaped to occasionally ovoid in appearance, with deep purple nuclei and colourless to very pale blue cytoplasm. Morphology of thrombocytes appeared to be consistent within individual smears but varied considerably between individuals. Distinguishing ovoid thrombocytes from lymphocytes was difficult, particularly in areas where staining was indistinct, e.g. near the head of the smear. Differentiation of lymphocytes and thrombocytes was not enhanced by staining with TB at pH 9; staining results were comparable with LG.

2.3.3.5 Monocytes

Monocytes were not observed in examined smears.

2.3.4 Fluorescent staining evaluation

In 50% of specimens, SSC vs. FL1 dot plots of whole blood suspensions incubated with low levels (25 to 50 nM) DiOC₆(3) yielded five resolved event populations (P1 to P5) as presented in Fig. 2.3A. With increasing dye concentration, events within P4 and P5 appeared to maintain relatively constant fluorescent intensity values, as indicated by the positions occupied on the FL1 channel axis. However, P1, P2 and P3 appeared to increase in fluorescent intensity with increasing dye dosage (Fig. 2.3A & B), to the extent that P3 and P4 appeared to merge at \approx 500 nM DiOC₆(3) (Fig. 2.3C). In some specimens, P3 could not be resolved, irrespective of DiOC₆(3) concentration (Fig. 2.3D, E & F).

Fluorescence-activated cell sorting was performed on specimens exhibiting resolved populations P1 to P5 (n = 5). P1 consisted of erythrocytes (Plate 2.3A & B). A number of these cells were found to be positive to BCB testing. P2 was also found to consist of erythrocytes (Plate 2.3C). P2 cells were morphologically similar to P1 cells, but exhibited a slightly mottled translucency; no BCB positivity was detected in P2 cells. P3 appeared to consist of thrombocytes, based on the ovoid to round morphology, purple staining of nuclear chromatin and colourless to very faint blue staining of cytoplasm (Plate 2.3D). Many of these cells were observed to have been damaged during the isolation process, and the spindle shape of thrombocytes seen in smears was not preserved. P4 appeared to consist of lymphocytes, based on the high nucleocytoplasmic ratio and basophilic staining of the cytoplasm (Plate 2.3E). Cells similar in appearance to P3 were occasionally observed and it was generally difficult to distinguish the two cell types by any means other than cytoplasmic staining (Plate 2.3F). Treatment of cell isolates with TB at pH 9 did not enhance resolution of thrombocytes and lymphocytes based on cytoplasmic staining. P5 was observed to consist of granulocytes (Plate 2.3G). Based on strong positivity of smear granulocytes to myeloperoxidase testing, sorted cells were tested using this procedure as a validation test but, surprisingly, were found to yield negative results (Plate 2.3H).

Cytotoxic effects of increased dosage of DiOC₆(3) on blood cells, as indicated by Sytox[®] Red positivity, were observed using FC. Effects of dye

dosage on RBC and WBC are summarised in Fig 2.4. Statistically significant dosage effects ($P < 0.05$, ANOVA) were observed above 500 nM for RBCs and 250 nM for WBCs, and mean proportional losses of cell viability were higher for WBCs than RBCs. At the maximum dosage level of 1,000 nM, 0.6% of RBCs were compromised and 80% of WBCs. Fig. 2.5 indicates the cytotoxic effects of increased dye dosage on cells, as indicated by Sytox[®] Red positivity, on SSC vs. FL1 dot plots. No discernible effect was observed at 50 nM (Fig. 2.5 A). Cells in the thrombocyte/lymphocyte population were compromised in the 250 to 500 nM range (Fig. 2.5 B & C). The effects on the FSC properties of cells, typically indicative of cell size, are presented in Fig. 2.6 on SSC vs. FSC dot plots. No discernible effect was observed at 50 nM dye (Fig. 2.6 A). At 250 to 500 nM (Fig. 2.6 B & C), the FSC signal of leukocytes became less diffuse and non-viable cells attained a lower FSC signal. At 1000 nM, most leukocytes were compromised, with similarly reduced FSC signal intensity (Fig. 2.6 D). A number of erythrocytes were also observed to attain a lower FSC value, but without loss of viability.

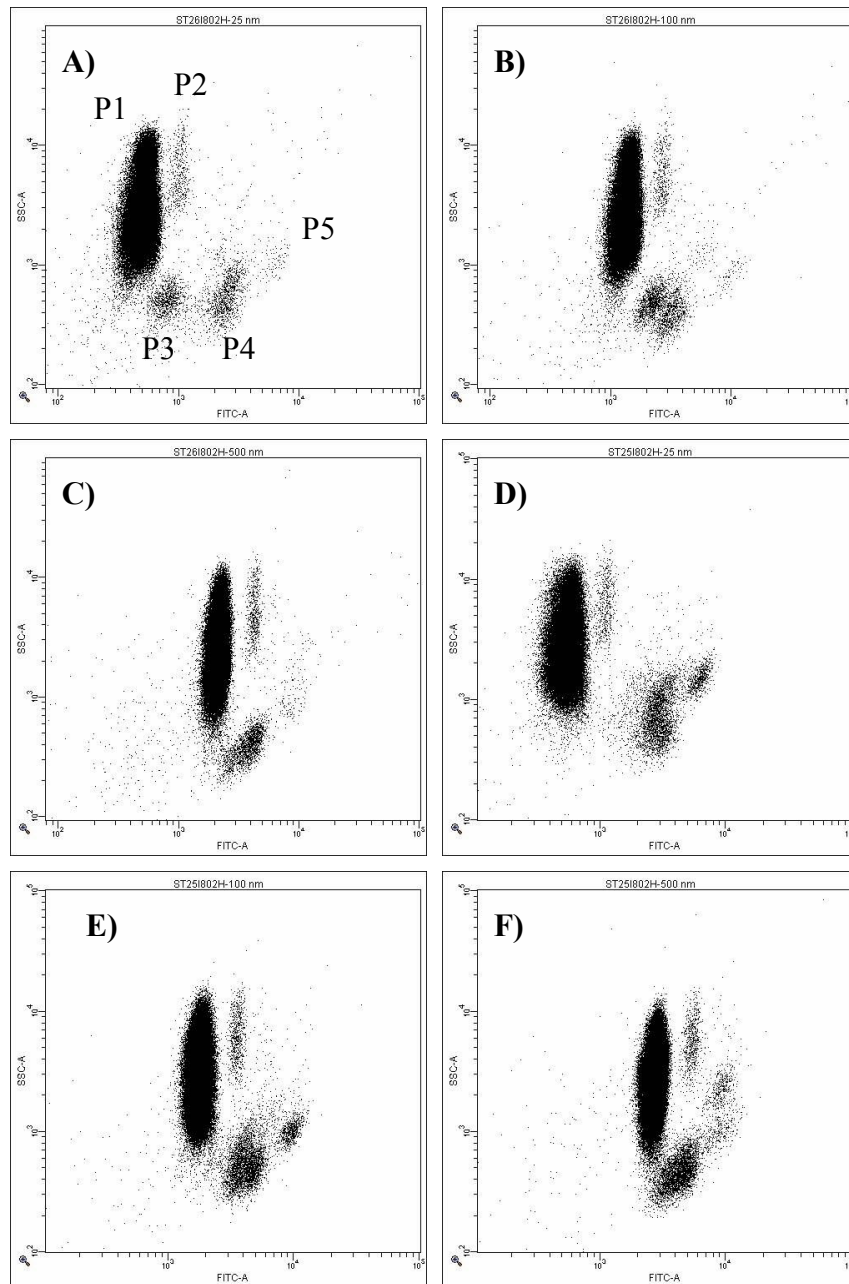


Figure 2.3. Flow cytometric side scatter (SSC) vs. 3,3'-dihexyloxycarbocyanine iodide fluorescence (FITC-A) dot plots for rainbow trout blood treated with 25 to 1000 nM dye. In 50% of samples, five cell populations (P1 to P5) were observed at 25 nM dosage (A). P1, P2 and P3 shifted to notably higher fluorescence intensities with increasing dye concentration, while P4 and P5 only exhibited slight increases (B). P3 and P4 were observed to merge at ≈ 500 nM (C). In some specimens, P3 did not appear to be resolved, irrespective of dye dosage (D, E & F).

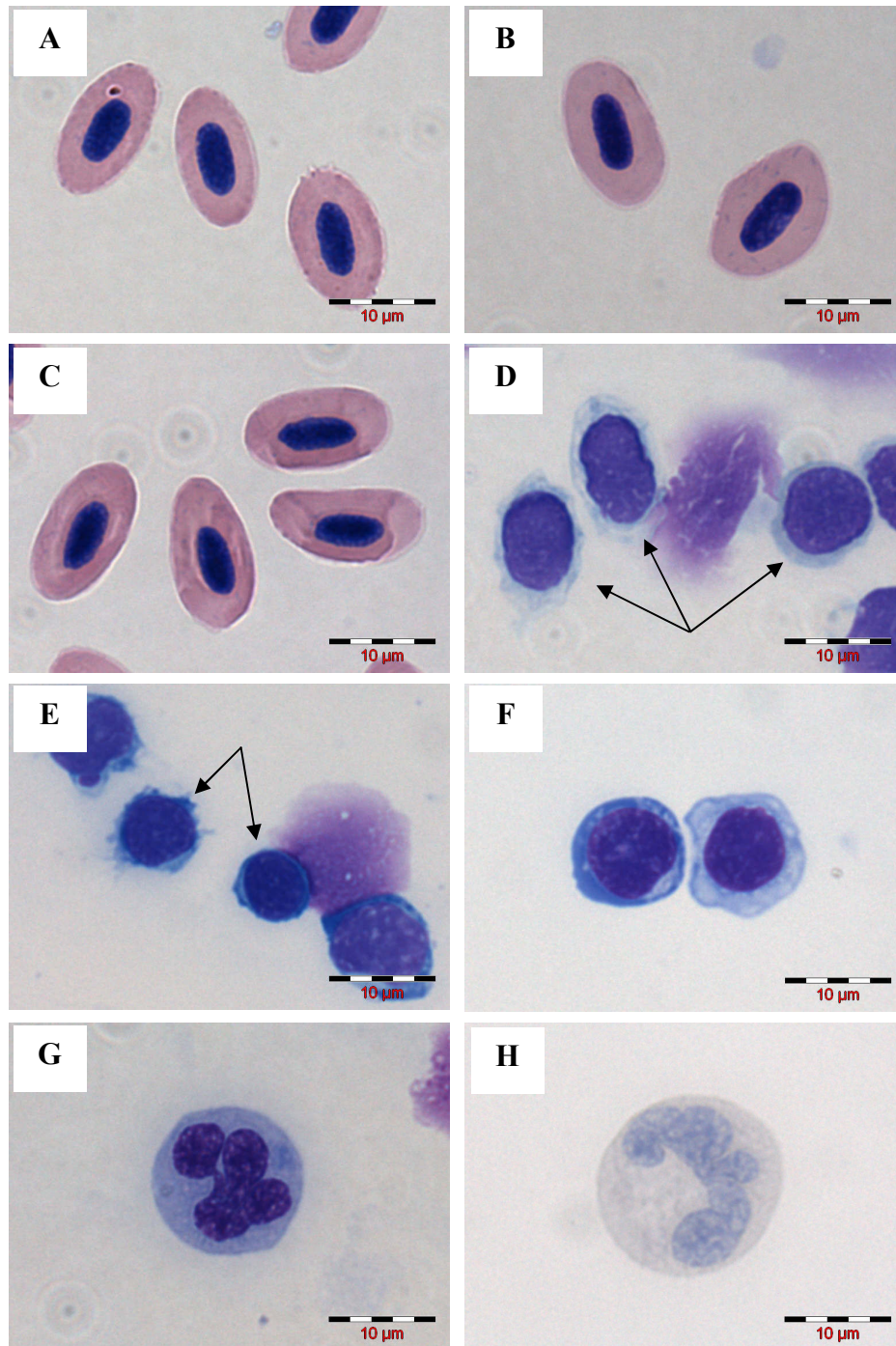


Plate 2.3. Cytocentrifuge preparations of sorted rainbow trout blood cells stained with Leishman's-Giemsa, including erythrocytes (A, B & C), thrombocytes (D), lymphocytes (E) and granulocytes (G). Lymphocytes (L) and thrombocytes (T) occasionally demonstrated morphological ambiguity (F). Granulocytes, which exhibited myeloperoxidase positivity in smears, yielded negative results in cytocentrifuge preparations (H).

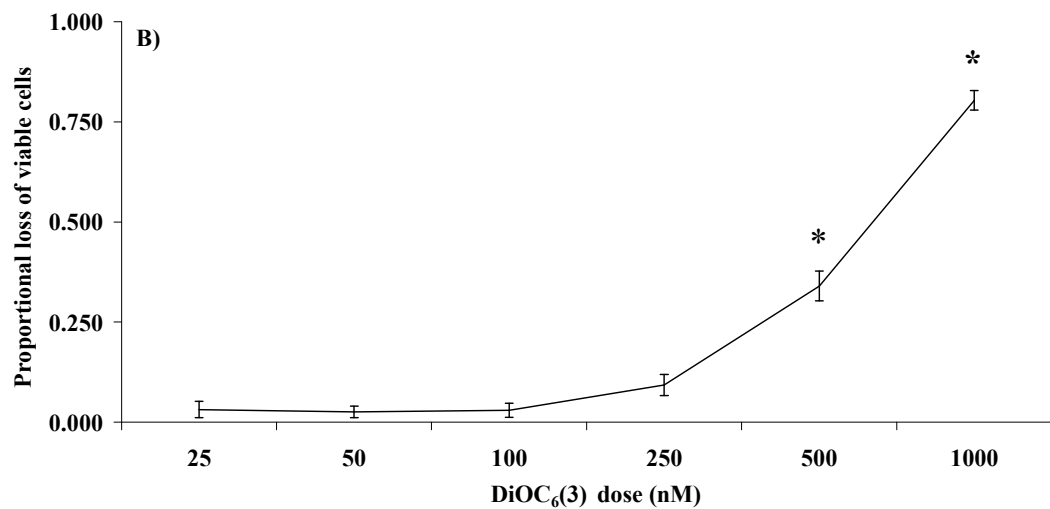
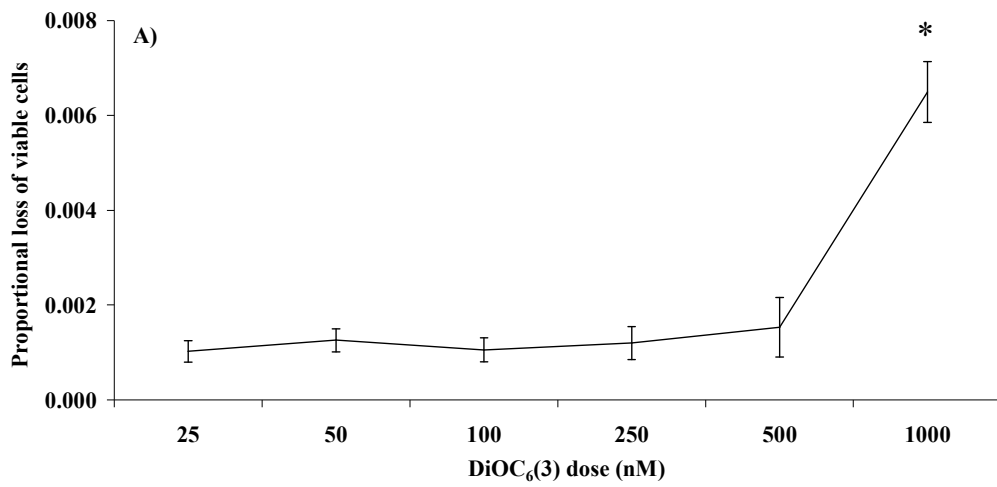


Figure 2.4. Cytotoxic effects of increased dosage of 3,3'-dihexyloxocarbocyanine iodide (DiOC₆(3)) on rainbow trout erythrocytes (A) and leukocytes (B), indicated by Sytox[®] Red positivity using flow cytometry. Significant toxic effects ($P < 0.05$, Kruskal-Wallis multiple comparisons test) are indicated with asterisks. $n = 10$ in all tests. Values are mean \pm SEM.

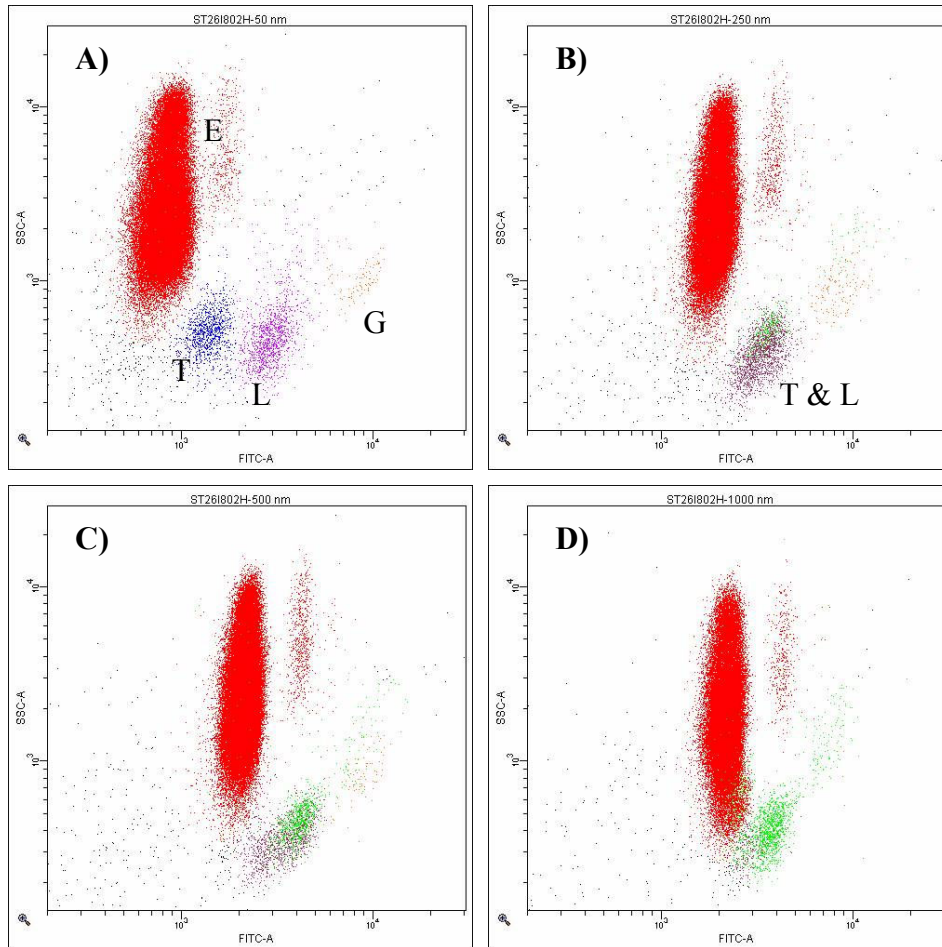


Figure 2.5. Flow cytometric side scatter (SSC) vs. 3,3'-dihexyloxcarbocyanine iodide (DiOC₆(3)) fluorescence (FITC-A) dot plots of rainbow trout blood cells treated with 50 to 1000 nM dye, indicating erythrocytes (e), thrombocytes (t), lymphocytes (l), granulocytes (g) and Sytox[®] Red-positive events (non-viable cells) indicated in green.

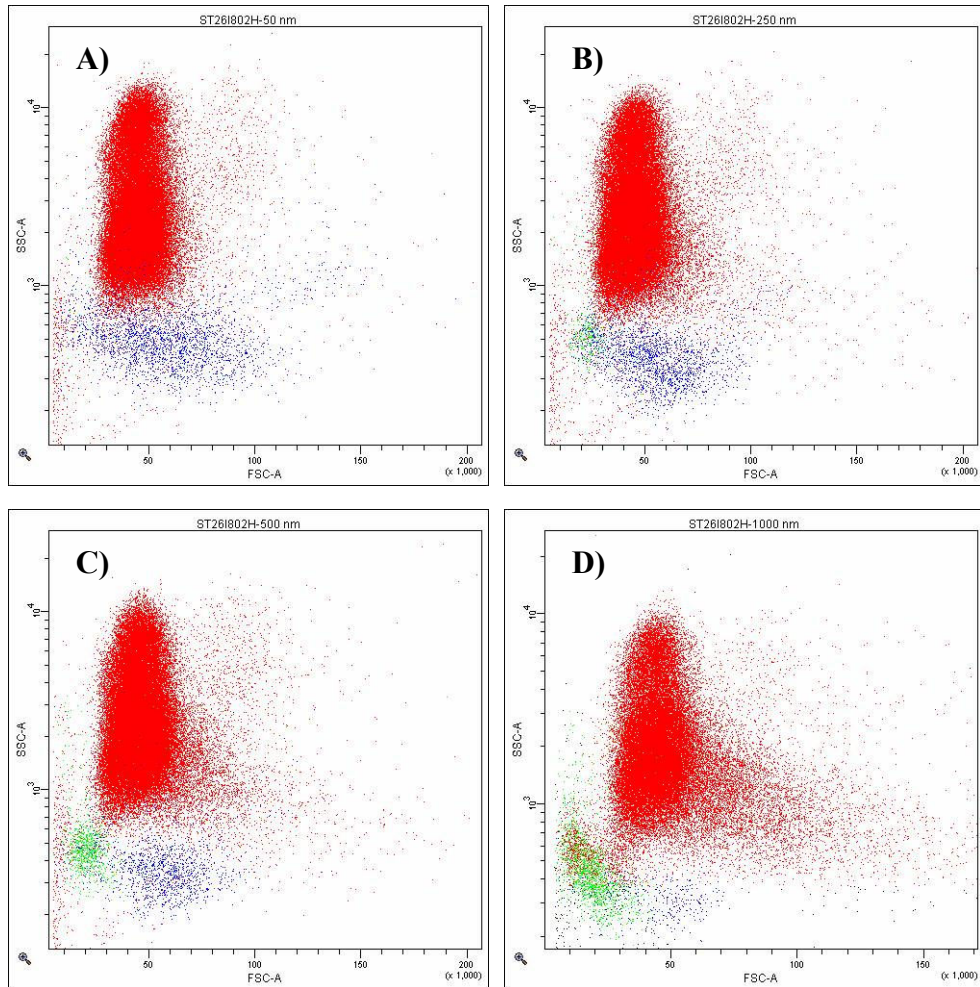


Figure 2.6. Flow cytometric side scatter (SSC) vs. forward scatter (FSC) dot plots of rainbow trout blood cells treated with 50 to 1000 nM 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3)), with erythrocytes indicated in red, leukocytes in blue and Sytox[®] Red-positive events (non-viable cells) indicated in green.

2.3.5 Flow cytometric count validations with fluorescent dye only

Comparative total RBC on yearling trout ($n = 10$), using the traditional manual methods and FC analysis with 50 nM DiOC₆(3), were found to be significantly different (Fig. 2.4). Mean RBCC were significantly higher ($P < 0.05$, paired T-test) using FC analysis.

FC resolution of thrombocytes, achieved with yearlings using 50 nM DiOC₆(3) in Section 2.3.4, could not be replicated with blood from individuals in this sample set. Irrespective of dye dosage, the thrombocyte population could not be completely resolved from the erythrocyte population (Fig. 2.8). Accordingly, total WBC and differential counts were not performed on this sample set.

FC resolution of thrombocytes, achieved with yearlings using 50 nM DiOC₆(3) in Section 2.3.4, could not be replicated with blood from individuals in this sample set. Irrespective of dye dosage, the thrombocyte population could not be completely resolved from the erythrocyte population (Fig. 2.8). Accordingly, total WBC and differential counts were not performed on this sample set.

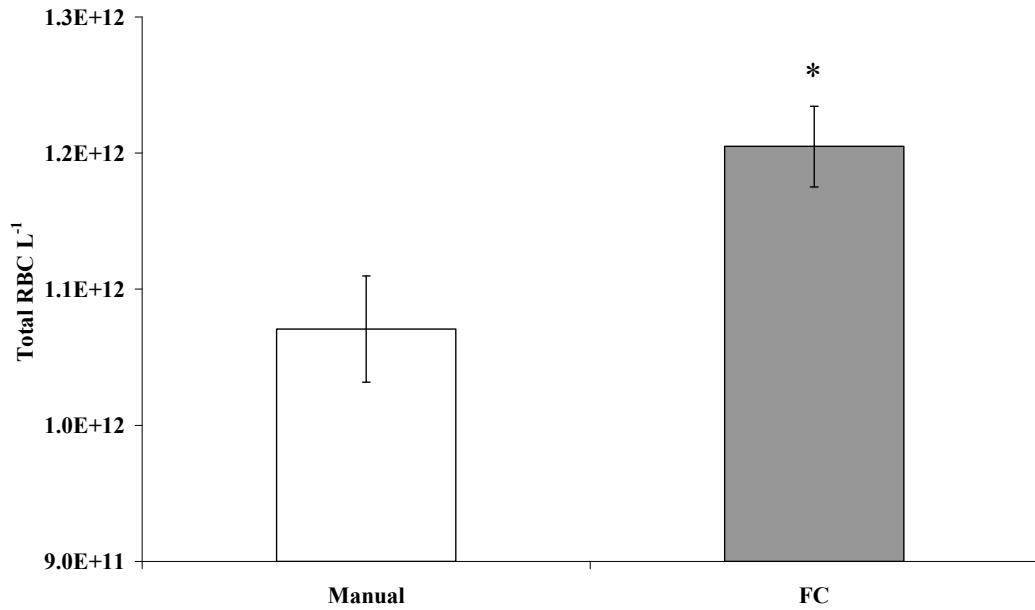


Figure 2.7. Mean (\pm SEM) total erythrocyte count (RBC L⁻¹) on rainbow trout whole blood using manual methods and flow cytometry with 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3)). Significant differences in counts ($P < 0.05$, paired T-test) are indicated with an asterisk.

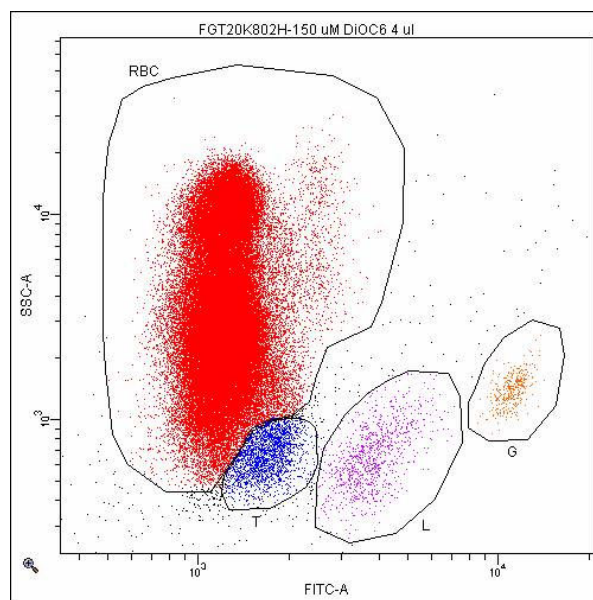


Figure 2.8. Flow cytometric side scatter (SSC-A) vs. 3,3'-dihexyloxycarbocyanine iodide (FITC-A) fluorescence dot plot of rainbow trout blood, showing poor resolution of thrombocytes (T) from erythrocytes (RBC) at 50 nM dye, consistent across an entire sample subset (n = 10).

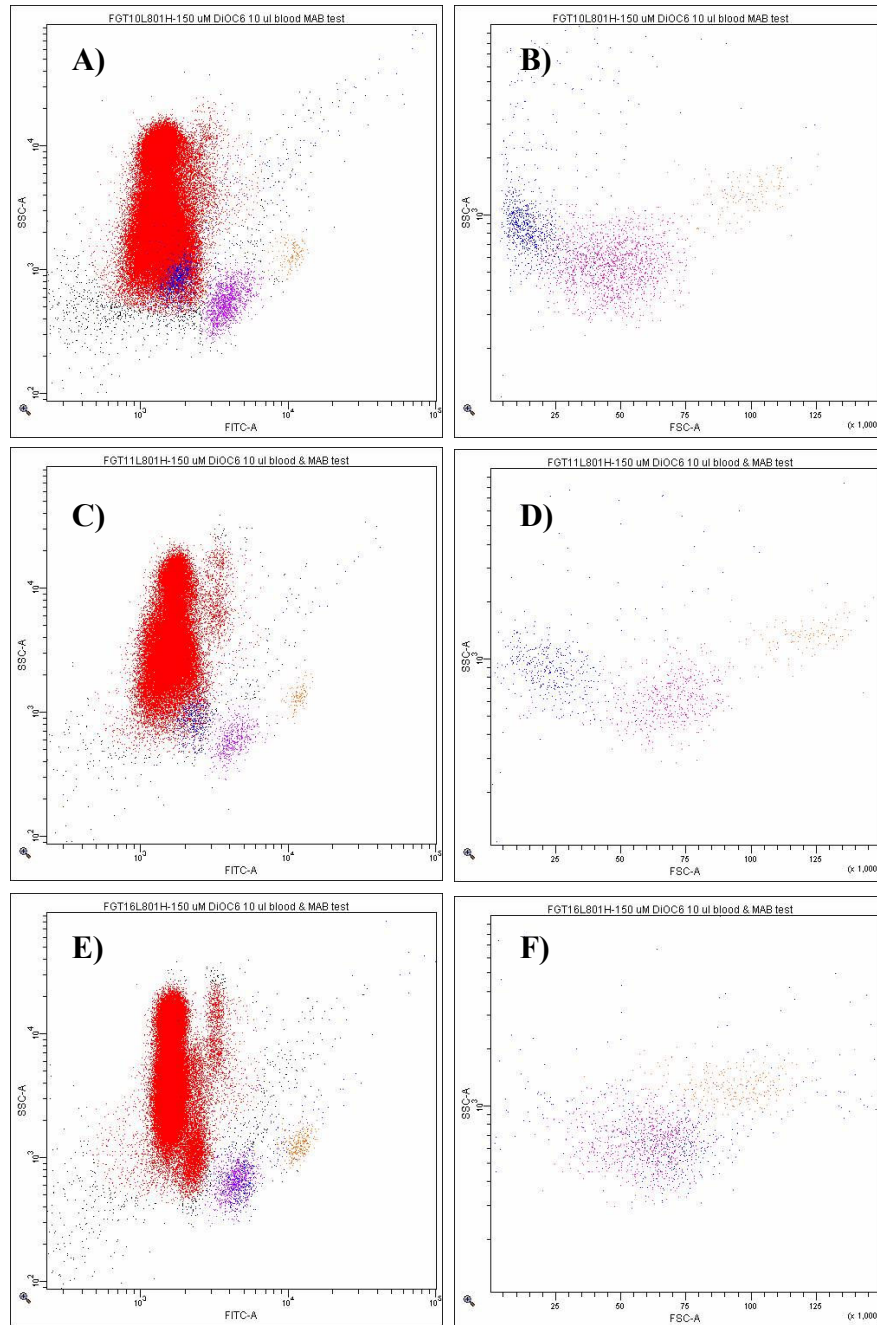


Figure 2.9. Flow cytometric dot plots of 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) fluorescence (FITC-A) and scatter data showing shifts in fluorescence intensity of thrombocytes (blue) in three specimens, at fixed dye dosage (50 nM) (A, C & E). The side scatter (SSC) vs. forward scatter (FSC) data for the gated leukocytes only (B, D & F for the three specimens, respectively) indicate trends in FSC similar to those observed in the fluorescence data.

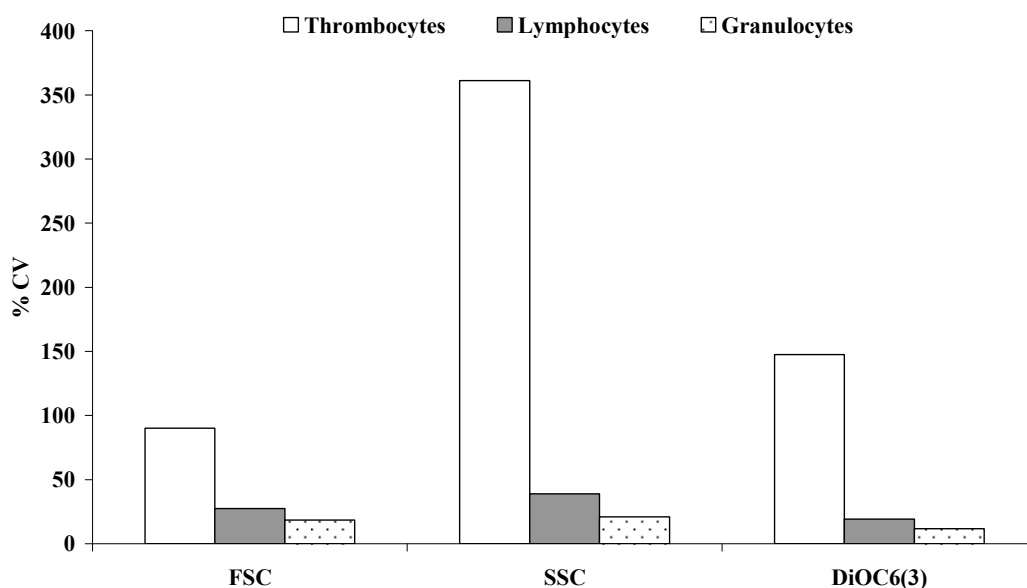


Figure 2.10. Mean percentage coefficient of variation (%CV) data for gated event parameters of leukocytes labelled with thrombocyte monoclonal antibody 42-allophycocyanin conjugate and counterstained with 50 nM 3,3'-dihexyloxacarboyanine iodide (DiOC₆(3)). Thrombocytes exhibited the most variation in terms of size (FSC), intracellular complexity (SSC) and DiOC₆(3) fluorescence.

2.3.6 Flow cytometric count validations with fluorescent dye and thrombocyte antibody

Yearling trout thrombocytes labelled with thrombocyte mAb 42-APC conjugate were identified on SSC vs FL1 dot plots of DiOC₆(3)-counterstained preparations using the multi-parameter functions within the DiVa software. Resolution of cell types into the five distinct populations identified in Section 2.3.4 with 50 nM DiOC₆(3) was observed in 50% of specimens in this sample, as before. Labelling with mAb-APC conjugate confirmed the apparent fluctuations in thrombocyte population fluorescence peak intensity observed previously (Fig. 2.9 A, C & E). Examination of the scatter data (SSC vs. FSC) of the three leukocyte populations

suggested that shifts in FSC for thrombocytes were mirrored by shifts in FL1 value (Fig. 2.9 B, D & F).

Mean %CV values were determined automatically by the DiVa software on gated events within each scatter/fluorescence parameter (Fig. 2.10), and were considerably higher for thrombocytes than lymphocytes and granulocytes, and highest for SSC values.

Significant differences ($P < 0.05$, Tukey's HSD post-hoc test) in mean total RBC were observed between the mAb+DiOC₆(3) method and counts obtained using both the manual method and samples stained with DiOC₆(3) alone (Fig. 2.11 A). Total WBC and differential leukocyte counts using DiOC₆(3) alone were not determined due to the inability to completely resolve the leukocyte populations in 50% of the samples. No significant differences in total WBC were detected between the manual method and the mAb+DiOC₆(3) method (Fig. 2.11 B). Significant method differences ($P < 0.05$, Wilcoxon Matched Pairs Test) in differential leukocyte counts were observed between the manual and mAb +DiOC₆(3) methods (Fig. 2.12). The manual method yielded a lower thrombocyte count and higher lymphocyte count. Granulocyte counts were comparable for the two methods.

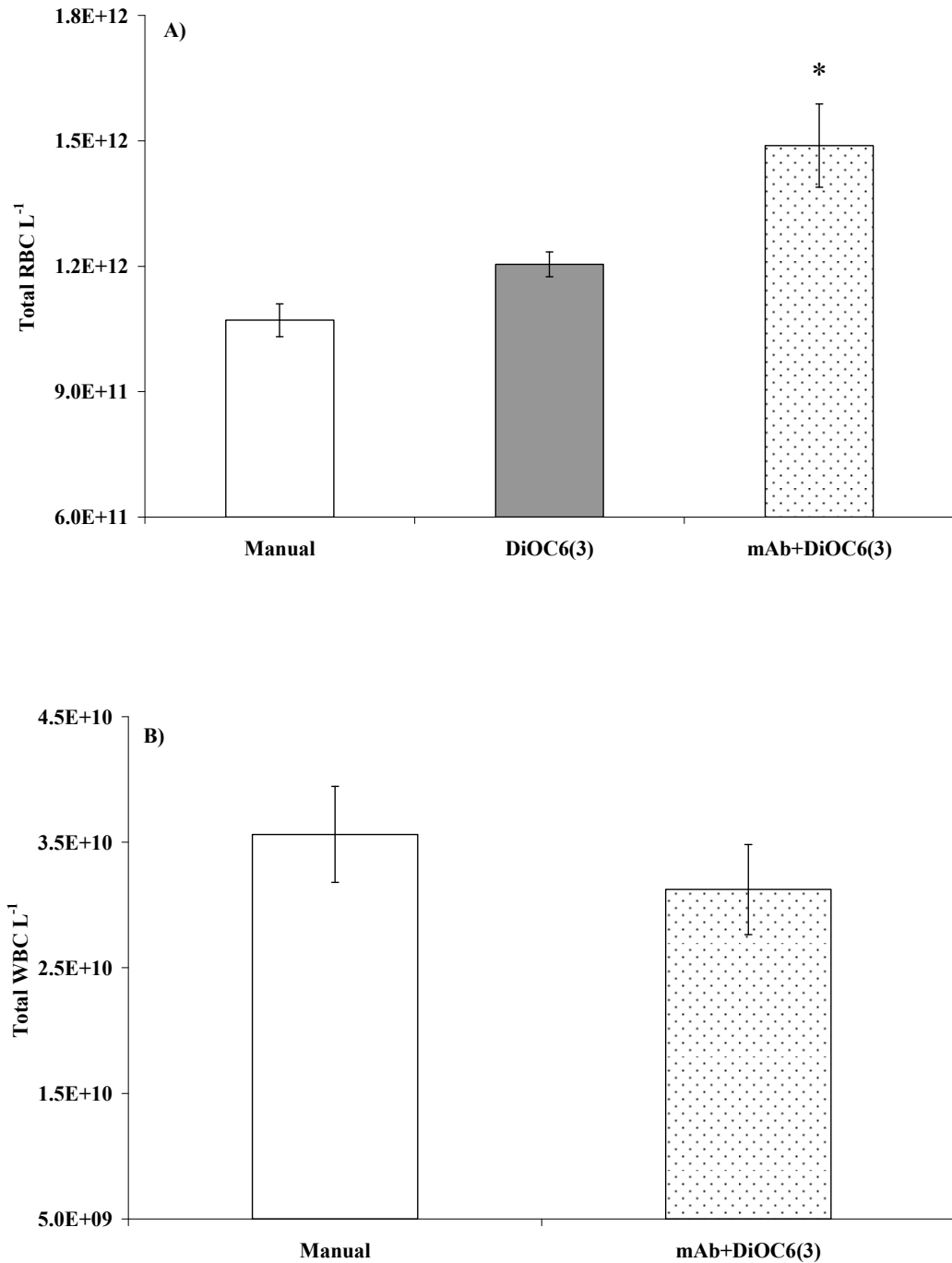


Figure 2.11. Comparative total erythrocyte counts (RBC L⁻¹; A) and total leukocyte counts (WBC L⁻¹; B) on rainbow trout (n = 10) using manual methods, 50 nM 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3)) and thrombocyte mAb +allophycocyanin conjugate+DiOC₆(3) counterstaining procedures. Significant differences (P < 0.05, Tukey's HSD post-hoc test) are indicated with asterisks. Values are mean ± SEM.

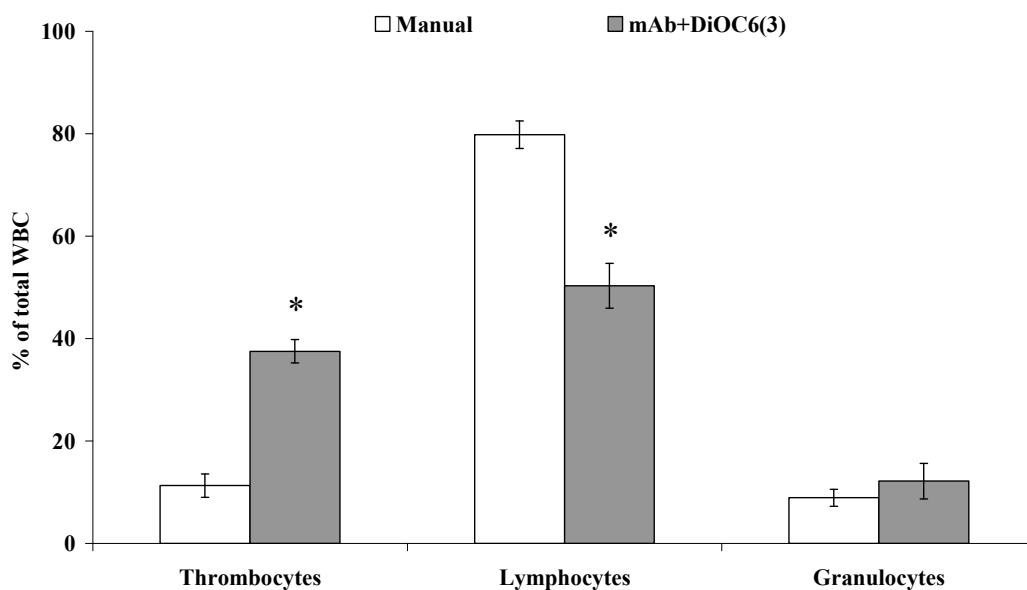


Figure 2.12. Differential leukocyte counts (DWBC) on rainbow trout (n = 10) using manual methods, 50 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) and thrombocyte mAb+allophycocyanin conjugate+DiOC₆(3) counterstaining procedures. Significant differences (P < 0.05, Wilcoxon matched pairs test) are indicated with asterisks. Values are mean ± SEM.

2.4 Discussion

This study confirmed that heparin is preferable to neutralised EDTA as an anticoagulant in the quantitative analysis of rainbow trout whole blood. IsoTon[®] II, a commercial saline solution for automated human blood cell enumeration, was found to be incompatible with heparin-treated trout blood. Flow cytometry was found to be a rapid, accurate and precise method for characterising, enumerating and sorting trout blood cells. Total RBC determined using FC were statistically significantly higher than manual counts, but the results were found to be generally comparable. Accurate total WBC and DWBC counts using FC and DiOC₆(3) necessitated the use of a thrombocyte mAb to resolve cell populations, resulting from apparent changes in thrombocyte morphology between individuals.

The current study indicated that Hct of heparin and EDTA-anticoagulated blood did not differ significantly after 2 hr on ice, but EDTA-treated blood Hct was significantly lower immediately after sampling (T0). An effect on cell size was also observed in blood smears prepared at T0. The relative Hct values at T0 and T120 suggest that EDTA-treated blood cells experienced shrinkage on sampling, but that cell volume subsequently normalised during the 2-hr incubation period. The changes in erythrocyte volume could possibly be attributed to the relatively high osmolality of the EDTA anticoagulant inducing hypertonic shrinkage of cells, particularly in the blood immediately in contact with the anticoagulant for the brief period before mixing. The significant increase in plasma Hb in the EDTA-treated blood indicated that erythrocyte lysis also occurred during the incubation period. Results appeared to support the findings of Bain and Bates (2001), who claimed that EDTA anticoagulant in excess of 1.5 mg mL⁻¹ may yield a falsely lower Hct. Korcock *et al.* (1988) and Hattingh (1975) reported haemolysis effects of EDTA over time, and Korcock *et al.* (1988) found that use of EDTA in combination with MS222 anaesthesia particularly favoured haemolysis. The results appear to support the general observation that heparin is the preferred anticoagulant for fish blood (Hattingh, 1975; Smit and Hattingh, 1980; Smit *et al.*, 1977). It has been argued, however, that heparin does not prevent clotting if coagulation has already initiated, that leukocytes are observed to clump in heparin-treated blood, and that background staining occurs with Romanowsky stains (Campbell and Ellis, 2007b). It has also been suggested that optimal choice of anticoagulant in terms of preservation of cell morphology is species-specific (Hrubec and Smith, 2000).

The LG staining protocol, though comparable to MGG, was less prone to batch variation in staining quality. The MGG protocol required control of the differential staining pattern in the final washing step by inspection of wet slides, whereas the LG procedure incorporated a final washing step with buffered water for a fixed period. Wright-Giemsa did not give the intensity of cytoplasmic staining of leukocytes necessary for morphological differentiation. Based on the combined results of the anticoagulant and Romanowsky staining tests, heparin and LG were used in all subsequent investigations.

Several authors have recommended the use of Cortland's saline for use with the blood of salmonids, based on its preservation of red blood cell mechanical properties for Coulter counter analysis (Hughes *et al.*, 1986) and the successful growth of a trout cell line in a medium consisting predominantly of the saline (Wolf, 1963). However, the use of saline solutions without calcium and magnesium, both present in Cortland's saline, is generally recommended for use in automated cell counters, as these metals promote cell clumping (Grogan and Collins, 1990) which may result in enumeration errors. Hughes *et al.* (1986) compared the effects of Cortland's saline and IsoTon[®] II saline (Coulter, USA) on the microscopic appearance of rainbow trout erythrocytes; cells diluted in Cortland's maintained their ellipsoidal shape for a longer period than IsoTon[®] II. This is especially interesting, as IsoTon[®] II is a product specifically formulated and recommended by instrument manufacturers for use as a sheath fluid in automated cell counters, albeit for mammalian samples. Calcium and magnesium-free phosphate buffered saline (PBS) is similarly recommended as an alternative sheath fluid. The comparative effects of both IsoTon[®] II and PBS on fish blood cells were thus relevant to this study, as the calcium and magnesium-free saline formulation least likely to compromise cellular integrity would be the obvious choice for use as a diluent and sheath fluid. PBS was found to be hypotonic and IsoTon[®] II hypertonic to the mean plasma osmolality. A significantly higher level of plasma Hb was measured in blood diluted with IsoTon[®] II, indicating a significantly higher degree of lysis. The apparent incompatibility of IsoTon[®] II with trout blood is supported by Hughes *et al.* (1986) who observed that trout erythrocytes lost their ellipsoidal shape after 15 min in this medium. IsoTon[®] II contains di-sodium EDTA (0.38 g L⁻¹), previously observed in this study to be incompatible with trout blood. It is suggested that osmotic effects on the cell membrane, in the presence of EDTA, may have enhanced cellular fragility of erythrocytes diluted with IsoTon[®] II. The study emphasised the importance of evaluating compatibility of sampling and analytical reagents and protocols, developed on mammalian blood, with fish blood.

Morphological and cytochemical characteristics of the blood cells generally conformed to those described in the literature (Blaxhall and Daisley, 1973; Fange, 1992; Campbell and Ellis, 2007b). The small erythrocyte sub-

population (P2) identified by FC was initially thought to consist of reticulocytes based on the similar proportion (i.e. $\pm 1\%$ of total erythrocytes) previously reported for brown trout (*Salmo trutta*; Blaxhall and Daisley, 1973). However, FACS-sorted and BCB-stained cells in this group were negative for residual RNA. The mottled translucent appearance of the cells may suggest that cells were compromised, although this conclusion is not supported owing to the lack of Sytox[®] Red staining. Thus, the exact nature of this group of erythrocytes is unclear. However, the negative test for immature erythrocytes suggests that these may be senescent cells.

Romanowsky and cytochemical staining properties of the granulocytes in this study were similar to those observed for human neutrophils, which are generally considered to be analogous to the predominant teleost granulocyte (Campbell and Ellis, 2007b). The granulocytes from blood films were moderately to very positive for myeloperoxidase, while FACS-sorted granulocytes were negative for myeloperoxidase. This is partly in contrast to the work of Zinkl *et al.* (1991) who found that rainbow trout “neutrophils” tested negative for peroxidase, although the specific peroxidase test was not stated, and therefore comparisons between studies are difficult to make. Myeloperoxidase has been observed to catalyse nitration of residual tyrosine in BSA (Sampson *et al.*, 1998), and the presence of excess BSA during FC sorting and collection may have inhibited the cytochemical detection of myeloperoxidase in the cytocentrifuged isolates. Although this was the only cytochemical test evaluated for characterisation of sorted granulocytes, the possible non-specific interference by BSA isolating medium in other protocols is worth noting.

Eosinophilic and basophilic granulocytes were not observed in this study, as also previously reported for rainbow trout (Zinkl *et al.*, 1991), although eosinophilic granulocytes have been anecdotally observed in blood smears prepared from wild rainbow trout (Landman pers. comm.). The eosinophils are generally easily distinguished by an intense eosinophilic response of cytoplasmic granules to Romanowsky stain. The negative reaction of granulocytes to TB at pH 3 (Tavares-Dias, 2006a) substantiated the lack of basophils. In addition to the general absence of eosinophils and basophils, there are conflicting reports

regarding the presence of circulating monocytes in rainbow trout (McCarthy *et al.*, 1973; Zinkl *et al.*, 1991). The Romanowsky-stained monocytes of fish typically possess agranular blue-grey cytoplasm, which may be vacuolated or include pseudopodia, and test positive for non-specific esterase (Campbell and Ellis, 2007b). Vacuolation and non-specific esterase positivity were not evident in smear leukocytes of this study, and so monocytes were also determined to be absent.

Thrombocytes were generally present in the elongated or spindle-shaped conformation. However, ovoid to round thrombocytes were also present and were difficult to discern from lymphocytes, particularly in areas of the slide where the characteristic basophilic staining of lymphocyte cytoplasm was indistinct or where nuclei occupied almost the entire cytoplasmic volume. Discrimination of thrombocytes on the basis of eosinophilia of small cytoplasmic granules (Campbell and Ellis, 2007b) was not found to be reliable, as very small granules could not be easily discerned. Discrimination of thrombocytes and lymphocytes in FACS-sorted isolates was even more difficult. The elongated or spindle shapes of thrombocytes observed in smears were not preserved during the isolation process. Cells either presented in a more elliptical to round shape or were destroyed completely leaving poorly stained artefacts that could not be compared with intact cells. As thrombocytes and lymphocytes yielded negative results in the cytochemical tests evaluated, these were not suitable for subsequent identification. The eosinophilic metachromatic effect of staining of thrombocytes with TB at pH 9 (Tavares-Dias, 2006b) was not observed in rainbow trout blood smears or cell isolates, and could similarly not be used for differentiation. The variable nature of thrombocyte morphology due to activation state during clotting or stage of maturity, and ambiguity in discriminating these cells from lymphocytes, is well described (Saunders, 1968; Hrubec and Smith, 2000; Campbell and Ellis, 2007b). Ellis (1976) cautioned against the use of smears containing a significant portion of oval or round thrombocytes for differential counts. Absolute resolution of this morphological ambiguity between fish lymphocytes and thrombocytes is best achieved using electron scanning microscopy techniques, as employed by Blaxhall (1983) and Thuvander *et al.* (1987), but these are of no practical use in terms of performing quantitative differential counts.

The current study demonstrated that FC resolution of individual cell populations in rainbow trout peripheral blood was optimal at dosages of DiOC₆(3) that did not compromise cell viability. The leukocytes appeared to be more susceptible than erythrocytes to toxic effects of DiOC₆(3) at concentrations above 250 nM. The erythrocytes and thrombocytes appeared to less readily absorb DiOC₆(3) at lower concentrations as judged by the relative increases in fluorescence intensity with increasing dye dosage. In all cell types, dye concentrations in excess of 250 nM did not result in appreciable increases in fluorescence, suggesting a level of saturation of available dye binding sites. A similar effect in mammalian cells has been previously reported at ≈2,000 nM DiOC₆(3) by Shapiro (2003b).

Thrombocyte affinity for DiOC₆(3), as indicated by fluorescence intensity, was found to be the most variable of the blood cell types and also coincided with variability in FSC properties. This variability was strongly reflected by the highest CV values for the thrombocytes. Shapiro (2003b) asserted that variation in fluorescence intensity is due to cell-to-cell variations in the number of binding sites. In this case, the rainbow trout thrombocytes demonstrated the most intracellular variation between samples, which may be linked to physiological changes. It is known that the thrombocytes become rounded in the activated state during clotting (Hrubec and Smith, 2000), a possible response to injury resulting from aggression against subordinates. Differences in physiological status are likely to occur due to social interaction in small groups of fish (Cooper et al., 1989; Pottinger and Pickering, 1992), such as those used in the current study. However, it is unclear if such physiological changes might induce cell swelling or alter dye adsorption properties of the cells. The thrombocytes in this study were observed to swell during FACS sorting and isolation. If such swelling occurred *in vivo* in response to stress, this could explain the variation in scatter and fluorescence parameters observed between individuals. The use of FC with conjugate-labelled mAb 42 and DiOC₆(3) confirmed the variations in thrombocyte morphology based on the mobility of this population within the scatter plots.

The mAb+DiOC₆(3) preparation yielded a higher total RBC than the manual and DiOC₆(3)-only methods. Possible sources of error here are potentially related to differences in the total number of cells counted between manual and FC methods (several hundred vs. several tens of thousands of cells, respectively) and differences in blood volumes used in the individual preparations (2 μ L vs. 10 μ L, representing a difference of $\approx 8 \times 10^6$ cells). Total WBC were found to be statistically similar for the manual and mAb+DiOC₆(3) methods, but differences in differential thrombocyte and lymphocyte counts were clearly evident. Results indicated a substantially lower proportion of lymphocytes in the FC counts, supporting similar results cited by Shapiro (2003b) of the comparison of FC and manual differential counts of human leukocytes. The opposite result was obtained by Morgan et al (1993), who obtained proportionally higher counts for lymphocytes with FC compared with manual counts. These results suggest that manual differentiation of thrombocytes and lymphocytes is either susceptible to morphological ambiguity which may result in over-estimation of lymphocytes, or inconsistent preservation of the morphological integrity of thrombocytes during the preparation of smears, as previously reported by Ellis (1976). Total WBC and DWBC obtained with the mAb+DiOC₆(3) cell preparation method were not significantly affected by the rigours of the centrifugation and washing steps. This suggests that use of FC with multiple, uniquely-labelled antibodies specific to the various leukocyte types may facilitate rapid, accurate and precise measurement of differential blood counts. Such an approach would eliminate the morphological ambiguity associated with non-specific staining using DiOC₆(3). The additional benefit of mAb's is the preservation of cell viability, which evidently may be compromised at the higher concentrations of DiOC₆(3) evaluated in the current study (>200 nM). This may be of particular importance when considering use of FACS-sorted cells for in vitro procedures.

Using rainbow trout as a model, this study demonstrated considerable potential of FC for rapid and accurate haematological assessment compared to traditional methods. The developed methodology emphasised and resolved the inherent ambiguity associated with differentiation of thrombocytes and lymphocytes in manual counts and was not compromised by variations in thrombocyte morphology. It is concluded that a combination of DiOC₆(3) and

fluorescent-labelled thrombocyte mAb is necessary for the complete differential leukocyte count. However, accurate total and partial differential counts can be obtained using DiOC₆(3) alone. It may thus be possible to obtain statistically robust information on multiple fish species within short time frames, providing an extremely valuable analytical tool in laboratory experiments and environmental effects-based assessments.

2.5 References

Ainsworth AJ. 1992. Fish granulocytes: morphology, distribution and function. *Annual Review of Fish Diseases* **2**:123-148.

Bain BJ, Bates I. 2001. Basic Haematological Techniques. In: Lewis SM, Bain BJ, Bates I, editors. *Dacie and Lewis Practical Haematology*. 9th ed. Edinburgh: Churchill Livingstone. p 19-46.

Bain BJ, Lewis SM. 2001. Preparation and staining methods for blood and bone marrow films. In: Lewis SM, Bain BJ, Bates I, editors. *Dacie and Lewis Practical Haematology*. 9th ed. Edinburgh: Churchill Livingstone. p 47-64.

Barham WT, Smit GL, Schoonbee HJ. 1980. The haematological assessment of bacterial infection in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **17**:275-281.

Barton BA, Iwama GK. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**:3-26.

Benfey TJ, Biron M. 2000. Acute stress response in triploid rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). *Aquaculture* **184**:167-176.

Blaxhall PC. 1972. The haematological assessment of the health of freshwater fish: a review of selected literature. *Journal of Fish Biology* **4**:593-604.

Blaxhall PC. 1983. Electron microscope studies of fish lymphocytes and thrombocytes. *Journal of Fish Biology* **22**:223-229.

Blaxhall PC, Daisley KW. 1973. Routine haematological methods for use with fish blood. *Journal of Fish Biology* **5**:771-781.

Bouck GR, Ball RC. 1966. Influence of capture methods on blood characteristics and mortality in the rainbow trout (*Salmo gairdneri*). *Transactions of the American Fisheries Society* **95**:170-176.

Campbell TW, Ellis CK. 2007a. Appendices and References. *Avian and Exotic Animal Hematology and Cytology*. Carlton: Blackwell Publishing Asia. p 239-277.

Campbell TW, Ellis CK. 2007b. Hematology of Fish. *Avian and Exotic Animal Hematology and Cytology*. Carlton: Blackwell Publishing Asia. p 93-111.

Casillas E, Smith LS. 1977. Effects of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology* **10**:481-491.

Chilmonczyk S, Monge D. 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. *Fish and Shellfish Immunology* **9**:319-333.

Cooper EL, Peters G, Ahmed II, Faisal M, Ghoneum M. 1989. Aggression in *Tilapia* affects immunocompetent leukocytes. *Aggressive Behavior* **15**:13-22.

Crippen TL, Bootland LM, Leong J-AC, Fitzpatrick MS, Schreck CB, Vella AT. 2001. Analysis of salmonid leukocytes purified by hypotonic lysis of erythrocytes. *Journal of Aquatic Animal Health* **13**:234-245.

Dacie JV, Lewis SM. 1991. Basic Haematological Techniques. *Practical Haematology*. 7th ed. Edinburgh: Churchill Livingstone. p 44-48.

Donaldson EM. 1981. The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering AD, editor. *Stress and Fish*. London: Academic Press. p 11-47.

Ellis AE. 1976. Leukocytes and related cells in the plaice *Pleuronectes platessa*. *Journal of Fish Biology* **8**:143-156.

Ellis AE. 1977. The leucocytes of fish: a review. *Journal of Fish Biology* **11**:453-491.

Ellsaesser CF, Clem LW. 1986. Haematological and immunological changes in channel catfish stressed by handling and transport. *Journal of Fish Biology* **28**:511-521.

Ellsaesser CF, Miller NW, Cuchens MA, Lobb CJ, Clem LW. 1985. Analysis of channel catfish peripheral blood leukocytes by bright-field microscopy and flow cytometry. *Transactions of the American Fisheries Society* **114**:279-285.

Fange R. 1992. Fish Blood Cells. In: Hoar WS, Randall DJ, Farrell AP, editors. *Fish Physiology*. London: Academic Press Ltd. p 2-54.

Grogan WM, Collins J. 1990. Preparation of single cell suspensions. *Guide to Flow Cytometry Methods*. New York: Marcel Dekker. p 22-28.

Hattingh J. 1975. Heparin and ethylenediamine tetra-acetate as anticoagulants for fish blood. *Pflügers Archiv* **355**:347-352.

Hesser EF. 1960. Methods for routine fish hematology. *Progressive Fish-Culturist* **22**:164-171.

Hoeger B, Koellner B, Kotterba G, Van den Heuvel MR, Hitzfeld B, Dietrich DR. 2004. Influence of chronic exposure to treated sewage effluent on the distribution of white blood cell populations in rainbow trout (*Oncorhynchus mykiss*) spleen. *Toxicological Sciences* **82**:97-105.

Houston AH, Dobric N, Kahurananga R. 1996. The nature of hematological response in fish. *Fish Physiology and Biochemistry* **4**:339-347.

Hrubec TC, Smith SA. 2000. Hematology of fish. In: Feldman BF, Zinkl JG, Jain NC, editors. *Schalm's Veterinary Hematology*. 5th ed. Baltimore: Wiley-Blackwell. p 1120-1125.

Hubl W, Wolfbauer G, Andert S, Thum G, Streicher J, Hubner C, Lapin A, Bayer PM. 1997. Toward a new reference method for the leukocyte five-part differential. *Cytometry* **30**:72-84.

Huffman PA, Arkoosh MR. 1997. Characteristics of peripheral blood cells from rainbow trout evaluated by particle counter, image analysis and hemocytometric techniques. *Journal of Aquatic Animal Health* **9**:29-39-248.

Hughes GM, Kikuchi Y, Barrington J. 1986. Physiological salines and the mechanical properties of trout red blood cells. *Journal of Fish Biology* **29**:393-402.

Inoue T, Moritomo T, Tamura Y, Mamiya S, Fujino H, Nakanishi T. 2002. A new method for fish leukocyte counting and partial differentiation by flow cytometry. *Fish and Shellfish Immunology* **13**:379-390.

Kent ML, Spitsbergen JM, Matthews JM, Fournie JW, Westerfield M 2007, *Diseases of Zebrafish in Research Facilities*, viewed 12.10.2007, [http://zebrafish.org/zirc/health/diseaseManual.php#Leishman's Giemsa Stain](http://zebrafish.org/zirc/health/diseaseManual.php#Leishman's%20Giemsa%20Stain)

Kfoury Jr JR, Kuroda A, Nakayasu C, Fukuda H, Okamoto N. 1999. Analysis of rainbow trout peripheral blood leucocytes separated by flow cytometry cell sorting. *Fish Pathology* **34**:1-6.

Korcock DE, Houston AH, Gray JD. 1988. Effects of sampling conditions on selected blood variables of rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **33**:319-330.

Mainwaring G, Rowley AF. 1985. The effect of anticoagulants on *Blennius pholis* L. leucocytes. *Comparative Biochemistry and Physiology* **80A**:85-91.

McCarthy DH, Stevenson JP, Roberts MS. 1973. Some blood parameters of the rainbow trout (*Salmo gairdneri* Richardson). *Journal of Fish Biology* **5**:1-8.

Morgan JAW, Pottinger TG, Rippon P. 1993. Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *Journal of Fish Biology* **42**:131-141.

Phi-Wilson JT, Recktenwald DJ 1994, *Coating agents for cell recovery*, viewed 7.9.2007, <http://www.freepatentsonline.com/5324629.html>

Pickering AD. 1986. Changes in blood cell composition of the brown trout, *Salmo trutta* L., during the spawning season. *Journal of Fish Biology* **29**:335-347.

Pickering AD, Pottinger TG. 1987. Crowding causes prolonged leucopenia in salmonid fish, despite interrenal acclimation. *Journal of Fish Biology* **30**:701-712.

Pottinger TG, Pickering AD. 1992. The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *Journal of Fish Biology* **41**:435-447.

Railo E, Nikinmaa M, Soivio A. 1985. Effects of sampling on blood parameters in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **26**:725-732.

Rehulka J, Adamec V. 2004. Red blood cell indices for Rainbow trout (*Oncorhynchus mykiss* Walbaum) reared in cage and raceway culture. *Acta Veterinaria Brno* **73**:105-114.

Roche H, Boge G. 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Marine Environmental Research* **41**:27-43.

Rowley AF. 1990. Collection, separation and identification of fish leukocytes. In: Stolen JS, Fletcher TC, Anderson DP, Robertson BS, van Muiswinkel WB, editors. *Techniques in Fish Immunology*. Fair Haven: S.O.S. Publications. p 113-136.

Sabnis RW, Todor GD, Madhukar NJ, Tukaram SD. 1997. DiOC₆(3): a useful dye for staining the endoplasmic reticulum. *Biotechnic and Histochemistry* **72**:253-258.

Sampson JB, Ye Y, Rosen H, Beckman JS. 1998. Myeloperoxidase and horseradish peroxidase catalyse tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Archives of Biochemistry and Biophysics* **356**:207-213.

Saunders DC. 1968. Variations in thrombocytes and small lymphocytes found in the circulating blood of marine fishes. *Transactions of the American Microscopical Society* **87**:39-43.

Scharsack JP, Steinhagen D, Leibold W, Rabe U, Korting W, Schuberth H-J. 2001. Flow cytometric analysis of mitogen-induced activation of rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Journal of Veterinary Medicine Series B* **48**:331-339.

Shapiro HM. 2003a. How flow cytometers work. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley & Sons, Inc. p 101-224.

Shapiro HM. 2003b. Overture. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley and Sons, Inc. p 1-60.

Shapiro HM. 2003c. Parameters and probes. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley & Sons, Inc. p 273-409.

Shapiro HM. 2003d. Using flow cytometers: applications, extensions and alternatives. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley & Sons, Inc. p 443-542.

Shaw AFB. 1930. A direct method for counting the leukocytes, thrombocytes and erythrocytes of birds' blood. *Journal of Pathology and Bacteriology* **33**:833-835.

Smit GL, Hattingh J. 1980. Haematological assessment of generally used freshwater fish blood anticoagulants. *Journal of Fish Biology* **17**:337-341.

Smit GL, Hattingh J, Schoonbee HJ. 1977. Observations on some effects of disodium ethylenediamine tetra-acetate and heparin on fish blood. *Comparative Biochemistry and Physiology* **57C**:35-38.

Sokal, R. R., and F. J. Rohlf. 1973. *Introduction to biostatistics*. W. H. Freeman and Company, San Francisco, CA, USA. Tavares-Dias M. 2006a. Cytochemical method for staining fish basophils. *Journal of Fish Biology* **69**:312-317.

Tavares-Dias M. 2006b. A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. *Journal of Fish Biology* **68**:1822-1833.

Thuvander A, Norrgren L, Fossum C. 1987. Phagocytic cells in blood from rainbow trout, *Salmo gairdneri* (Richardson), characterized by flow cytometry and electron microscopy. *Journal of Fish Biology* **31**:197-208.

Valenzuela AE, Silva VM, Klempau AE. 2007. Some changes in the haematological parameters of rainbow trout (*Oncorhynchus mykiss*) exposed to three artificial photoperiod regimes. *Fish Physiology and Biochemistry* **33**:35-48.

Waterstrat PR, Ainsworth AJ, Capley G. 1988. Use of a discontinuous Percoll gradient technique for the separation of channel catfish, *Ictalurus punctatus* (Rafinesque), peripheral blood leucocytes. *Journal of Fish Diseases* **11**:289-294.

Weinreb E. 1958. Studies on the histology and histopathology of the rainbow trout, *Salmo gairdneri* (Richardson). 1. Haematology: under normal and experimental conditions of inflammation in rainbow trout. *Zoologica NY* **46**:145-153.

Weyts FAA, Flik G, Rombout JHWM, Verburg-van Kemenade BML. 1998a. Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, *Cyprinus carpio* L. *Developmental and Comparative Immunology* **22**:551-562.

Weyts FAA, Flik G, Verburg-van Kemenade BML. 1998b. Cortisol inhibits apoptosis in carp neutrophilic granulocytes. *Developmental and Comparative Immunology* **22**:563-572.

Wojtaszek J, Dziewulska-Szwajkowska D, Lozinska-Gabska M, Adamowicz A, Dzugaj A. 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): Cortisol effect on the carp blood. *General and Comparative Endocrinology* **125**:176-183.

Wolf K. 1963. Physiological salines for fresh water teleosts. *The Progressive Fish-Culturist* **25**:135-140.

Woodward CC, Strange RJ. 1987. Physiological stress responses in wild and hatchery-reared rainbow trout. *Transactions of the American Fisheries Society* **116**:574-579.

Zinkl JG, Cox WT, Kono CS. 1991. Morphology and cytochemistry of leukocytes and thrombocytes of six species of fish. *Comparative Haematology International* **1**:187-195.

Chapter Three

Flow cytometric characterisation of freshwater crayfish haemocytes for the examination of physiological status in wild and captive animals

As accepted for publication.

Taylor, S., Landman, M. and Ling, N. (2009).

In Journal of Aquatic Animal Health.

3.1 Introduction

The haemolymph and haemocyte components of the arthropod circulation are responsible for various protective mechanisms ranging from coagulation and wound repair to cell-mediated immunity (Theopold *et al.*, 2004; Iwanaga and Lee, 2005; Jiravanichpaisal *et al.*, 2006). In crustacea, two major haemocyte groups comprised of typically agranular and granular cells are accepted (Hose *et al.*, 1990). These groups may be further differentiated into the hyaline haemocytes, and the semi-granular and granular haemocytes which possess some overlapping functional roles (Johansson *et al.*, 2000; Jiravanichpaisal *et al.*, 2006). Hemocyte counts may provide an indication of sub-acute physiological effect in crustaceans (Smith, 1991) and changes in hemocyte count has been shown to be a suitable indicator of stress in some species (Lorenzon *et al.*, 2001).

Greater understanding of species-specific physiology is important because physiological processes are known to vary considerably between invertebrate taxa. Much of our current understanding of the invertebrate immune system has arisen from the investigation of common research species or those of commercial importance. However, Jiravanichpaisal *et al.* (2006) suggested that there may be value in studying previously unexamined species. Recent interest has developed around the use of the New Zealand freshwater crayfish *Paranephrops planifrons* as a sentinel species in eutrophic environments as this species has diminished or disappeared from parts of its natural range in recent decades. One of the possible consequences of eutrophication is increased sediment manganese cycling and bioavailability (Bryant *et al.*, 1997; Tankere *et al.*, 2000; Baden and Neil, 2003) as this metal has been shown to impair hemocyte function in the Norway lobster (*Nephrops norvegicus*) (Hernroth *et al.*, 2004). Thus, declining water quality as a result of eutrophication poses a significant threat to all aquatic organisms, but may be of more immediate concern to already threatened species.

The haemocytes of the New Zealand freshwater crayfish have not previously been classified or examined as a tool for ecological assessment. Crustacean hemocyte classification has traditionally been based on morphology, cytochemistry and functional studies (Hose *et al.*, 1990). In many regards, flow cytometry (FC) has superseded microscopic evaluation as this technique offers the

ability to rapidly and simultaneously discriminate cellular populations based on a variety of parameters such as relative size, complexity and fluorescent properties (Shapiro, 2003). The technique has successfully been applied to differential hemocyte investigations and immune responses in several crustacean (e.g. Owens and O'Neill, 1997; Cardenas *et al.*, 2000; Yip and Wong, 2002; Cardenas *et al.*, 2004) and mollusc species (e.g. Xue *et al.*, 2001; Goedken and De Guise, 2004; Travers *et al.*, 2008).

The purpose of the current study was to identify and characterise the haemocytes of a freshwater crayfish species. Here we describe a generalised FC protocol for rapid hemolymph screening and objective haemocyte characterisation that was applied to the New Zealand freshwater crayfish. This method was subsequently used to obtain total and differential hemocyte counts to examine the physiological status of wild and captive animals.

3.2 Materials and methods

3.2.1 Animals

A total of approximately 50 crayfish, *Paranephrops planifrons* (19-51 mm ocular carapace length, 6-86 g total weight), were collected from three sites (N = 10-20 per site) in the Rotorua Lakes District of the central North Island, New Zealand. Crayfish were hand-collected by scuba divers from Lakes Rotorua (38° 6.29'S, 176° 14.85'E), Rotoiti (38° 3.20'S, 176° 25.86'E) and Rotoma (38° 2.57'S, 176° 34.96'E) during May 2008. Specimens were transported back to the laboratory in 20 L plastic pails within 1 h of capture and were anaesthetised on ice prior to size measurement and hemolymph sampling. An additional 10 Lake Rotoma specimens were retained in the laboratory, housed in 500 L flow-through tanks (200 L/h) at 12 ± 0.5°C, supplied with constant aeration and dechlorinated Rotorua city tap water, and maintained on a 16:8 h light:dark photoperiod. Laboratory-housed crayfish were fed every second day with a selection of chopped vegetables. Rocks were placed in tanks, providing refugia to minimise stress and aggression.

3.2.2 *Haemolymph sampling procedure*

For all assessments, 100 μL hemolymph samples were withdrawn dorsally from the pericardial sinus between the carapace and first abdominal segment using a 0.5 mL syringe fitted with a 27-gauge needle. A range of anticoagulants including Alsever's solution (Gibco, NZ), IsoTon II (Beckman Coulter, USA) and an established crustacean anticoagulant containing citrate and EDTA (Söderhäll and Smith, 1983), were examined and found to be incompatible with crayfish hemolymph for the purposes of this FC evaluation. Instead, syringes were preloaded with 100 μL of ice-cold hemolymph fixative solution (4% formaldehyde) so that samples were diluted 1:1 (v/v) to maintain cell integrity thereby preventing clotting and clumping of cells *in vitro*. Immediately following collection, the hemolymph samples were aspirated into pre-chilled 0.5 mL centrifuge tubes and gently mixed.

3.2.3 *Preliminary flow cytometric sample evaluation*

Hemolymph samples in fixative solution (200 μL) were transferred to pre-chilled 5 ml polypropylene cytometry tubes (Falcon, USA), previously coated with 4% bovine serum albumin (BSA; Sigma-Aldrich, Australia) in phosphate-buffered saline (PBS) for 3 h, and diluted to 1 mL with ice-cold PBS. Diluted samples were incubated on ice with 4 μL of 0.5 mg mL^{-1} 3,3'-dihexyloxycarbocyanine iodide DiOC₆(3) (Molecular Probes, USA) for 15 min in the dark. DiOC₆(3) is a lipophilic dye that stains the endoplasmic reticulum and is used in FC membrane potential measurements (Shapiro, 2003).

Sample analysis was performed on a FACSVantage SE FC, equipped with DiVa electronics and software (BD Biosciences, USA), using a 488 nm laser powered at 300 mW. Forward scatter (FSC), side scatter (SSC) and fluorescence in the 530/30 nm wavelength range (FL1) were measured. Detector photomultiplier voltages were 125, 300 and 250 mV respectively, and outputs were viewed in logarithmic mode. Threshold was adjusted between 200 and 5000 as required to exclude debris and enhance hemocyte resolution at high event counts. The instrument sheath fluid was PBS delivered at 69 kPa. Samples were

passed through a 70 μm nozzle with a flow rate of 1000 events/s. Between 10,000 and 120,000 events were recorded, depending on resolution between debris and hemocyte populations. Scatter plots (FSC vs. SSC) were generated for each treatment. Gating was performed using the DiVa software to define population subsets and to exclude unwanted events. Intact cellular populations corresponded with clusters of events with maximal DiOC₆(3) dye uptake, characterised by narrow, positive fluorescence peaks which were gated and then highlighted on scatter plots. The SSC parameter events associated with the fluorescence peak on the FITC parameter histogram were gated, and these events were similarly gated on the FSC histogram (Fig. 1A, B and C).

3.2.4 Sorting and characterisation of haemocytes

Cells were sorted based on the previously determined gates. Drop drive frequency and the drop delay were optimised as recommended by BD Biosciences (Becton-Dickinson, 2004a). Sorting was performed using a drop drive frequency setting of 22 kHz, with amplitude adjusted to 3V and a drop delay of 13 drops. Sorting purity was adjusted to the Yield Mask setting. Sorted cells were collected in BSA-coated 5 mL polypropylene tubes containing 3 ml PBS supplemented with 0.25% BSA. The tube holder was cooled with recirculating ice water. Collected cells were spun directly onto polylysine-coated Shandon Cytoslides using a Shandon Cytospin 4 centrifuge (Thermo Electron Corporation, USA). Cytoslides were fitted to BSA-coated cytofunnels with filter cards pre-wetted using 50 μL of PBS. Samples were centrifuged at 800 rpm for 3 min and allowed to air-dry for 30 min. Cells were fixed in absolute methanol for 5 min and subsequently stained with Leishman's-Giemsa. Slides were air-dried again and cover-slipped using Clarion mounting medium (Biomeda, USA). Images of representative cells were captured at 1000x magnification using an Olympus BX61 microscope equipped with a SIS ColorView III digital camera. Image analysis to determine cellular and nuclear dimensions was performed using AnalySIS software (LifeScience Series, Olympus, USA). Nucleocytoplasmic ratio was expressed as the percentage of the nuclear area (μm^2) relative to the cellular area.

3.2.5 Hemocyte evaluation in wild and captive crayfish

Total (THC) and differential hemocyte counts (DHC) were determined for wild and captive specimens (n = 10 per site or wild vs. captive). Accurate cell counts were determined using TruCount tubes (BD Biosciences, USA) containing approximately 50,000 fluorescent-labelled beads. Accurate bead counts per tube were stated on individual batches of tubes. One millilitre of PBS was added to a TruCount tube, vortexed, and 100 μ L aliquots (approximately 5000 beads) added to sample tubes prior to FC analysis. For each sample, a total of 250 beads were counted. Total or individual population cell counts were determined using the following formula:

$$\text{cells/mL} = \frac{\text{cells counted} \times \text{beads per tube} (\sim 5000)}{\text{beads counted} (250) \times \text{haemolymph volume} (0.1 \text{ mL})}$$

The DiVa software provides several options to view captured data as single parameter histograms, or as dual parameter plots of scatter or fluorescence signal intensity. Two parameters may be viewed as dot plots, where each dot represents an event displaying the corresponding value of each parameter. Alternatively, in contour plots a third dimension is provided by joining coordinates with similar event counts, as on a topographical map (Becton-Dickinson, 2004b). The “20% probability” software option was used for contour analysis, where the area between each contour line contained 20% of the total event count within each population gate such that the outermost contour represented 10% of the total number of events, the second contour 30%, then 50%, 70% and 90%. Gates were drawn, by visual inspection, around distinct event clusters on the dot plots. Gates were then spatially adjusted to yield concentric contours, ensuring that resolved populations did not overlap on the contour plots so that a minimum of 95% of the total event count was accounted for. Thus, a standardised methodology for gating the hemocyte populations was adopted for all samples.

3.2.6 Statistics

Statistical comparison of total and differential hemocyte counts between sites was performed using one-way analysis of variance (ANOVA) with site as a factor, followed by a Tukey's post-hoc test where significant site effects were observed. Comparisons of total and differential cell counts between Lake Rotoma specimens sampled immediately upon capture and after 14 d in captivity were also performed using ANOVA. Total hemocyte counts were log-transformed prior to analysis. Because differential counts were measured as proportions, these data were arcsine transformed prior to analysis (Sokal and Rohlf, 1973). All statistical analyses were performed using STATISTICA v8.0 software. The critical level of statistical significance for all tests was $\alpha = 0.05$.

3.3 Results

Hemocyte populations were successfully resolved on FSC vs. SSC data plots using DiOC₆(3) staining and scatter parameter gating. Fluorescence peaks could be gated on FL1 histograms (Fig. 3.1A) enabling identification and exclusion of broad bands of low intensity events consistent with debris and autofluorescence (Fig. 3.1B, C). Three event clusters were observed in the typical FSC vs. SSC hemogram (Fig. 3.2A). Gates were drawn around each event cluster (Fig. 3.2B) which were provisionally classified as hyaline (H), semigranular (SG) and granular (G) hemocyte populations based on scatter properties. Gates were manually adjusted to yield 20% probability contour plots (Fig. 3.2C) with differential counting and sorting performed using these gates.

From the defined and sorted FC populations, variations in cellular morphology and response to Leishman's-Giemsa staining were evident within each sorted subset, although some population overlap was evident based on scatter and 20% probability contour plots. Hyaline cells were characterised by a significantly lower cell area than the semigranular and granular cells (Plate 3.1A; Table 3.1), and the nuclear areas and nucleocytoplasmic ratios were found to be significantly different for the hemocyte types ($P < 0.05$, ANOVA). Hyaline cells had the highest nucleocytoplasmic ratio and granular cells the lowest. The

cytoplasm and nuclei of all cell types were basophilic to Leishman's-Giemsa stain. Nuclear staining of the hyaline cells varied between dark blue, typically in the smaller nuclei, and light purple in the larger. The sparse cytoplasm stained light blue with little evidence of granulation. The semigranular cell nuclei stained between light and dark blue, the darker colour generally associated with smaller nuclei (Plate 3.1B). Cytoplasmic granularity in these cells varied, from being barely discernible to clearly evident as a dense packing of unstained granules within a light blue cytoplasm. The granular cells were characterised by eccentrically located small, dark blue nuclei and faintly to strongly eosinophilic granules densely packed within a light blue cytoplasm (Plate 3.1C). Each subset also contained a small but unquantified proportion of cells similar in appearance to those characteristic of adjacent gates.

Significant site effects on THC or DHC were not observed between the three lake populations (Fig. 3.3A and 3.4A). Total hemocyte counts of Lake Rotoma specimens retained in the laboratory decreased significantly ($P < 0.05$) to approximately 37% of the initial count after 14 d in captivity (Fig. 3.3B). However, the DHC was not affected by reduced THC (Fig. 3.4B).

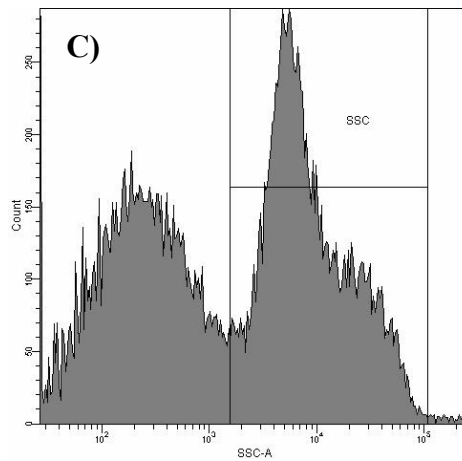
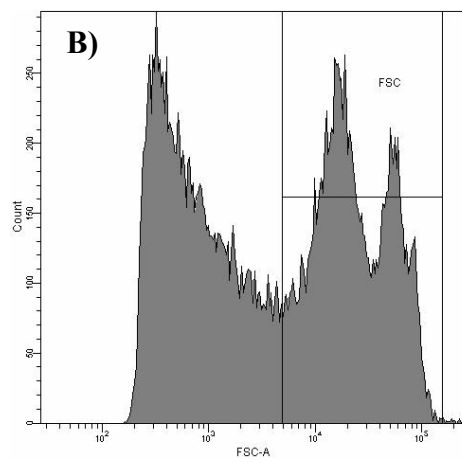
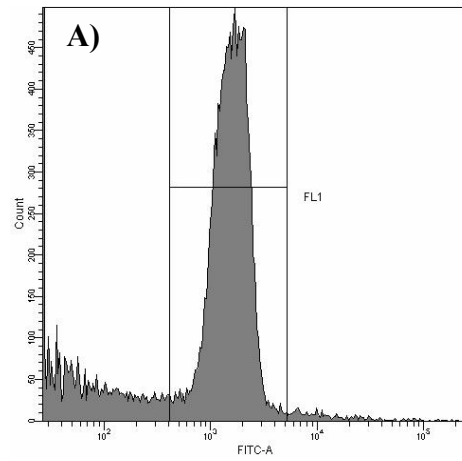


Figure 3.1. Gating procedure for haemocyte counts on 3,3'-dihydroxyoxycarbocyanine iodide (DiOC₆(3))-stained hemolymph samples. A) Fluorescent event (FL1) gate, B) forward scatter (FSC) subset of FL1 gate and C) side scatter (SSC) subset of the FSC gate.

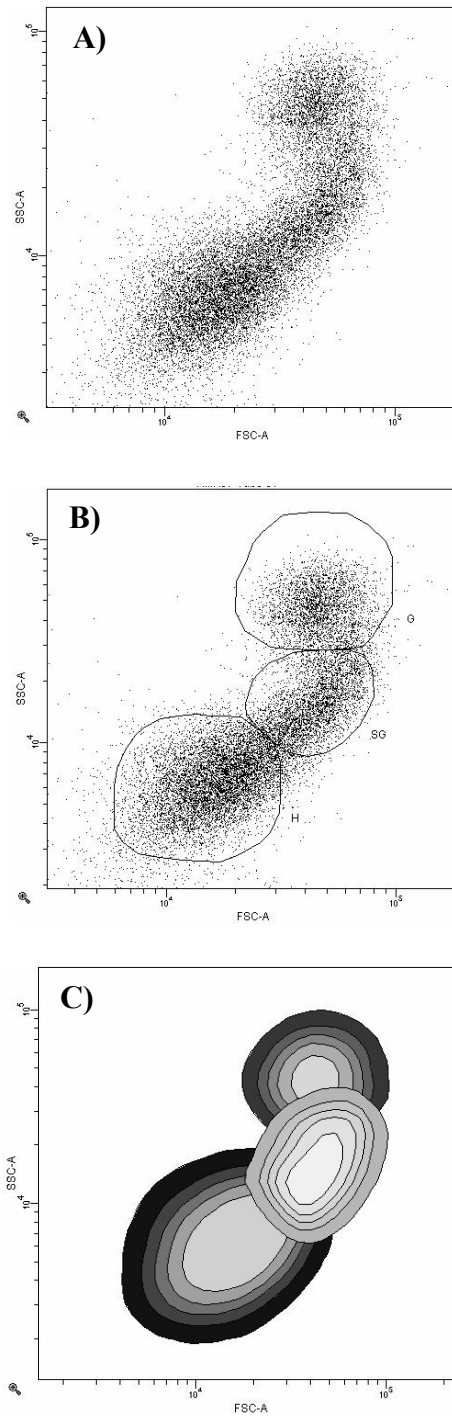


Figure 3.2. Representative forward scatter (FSC) vs. side scatter (SSC) data plots of hemolymph showing A) all cellular events, B) gating of individual hyaline (H), semigranular (SG) and granular (G) hemocyte populations and C) “20% probability” contour plots of hemocyte gates.

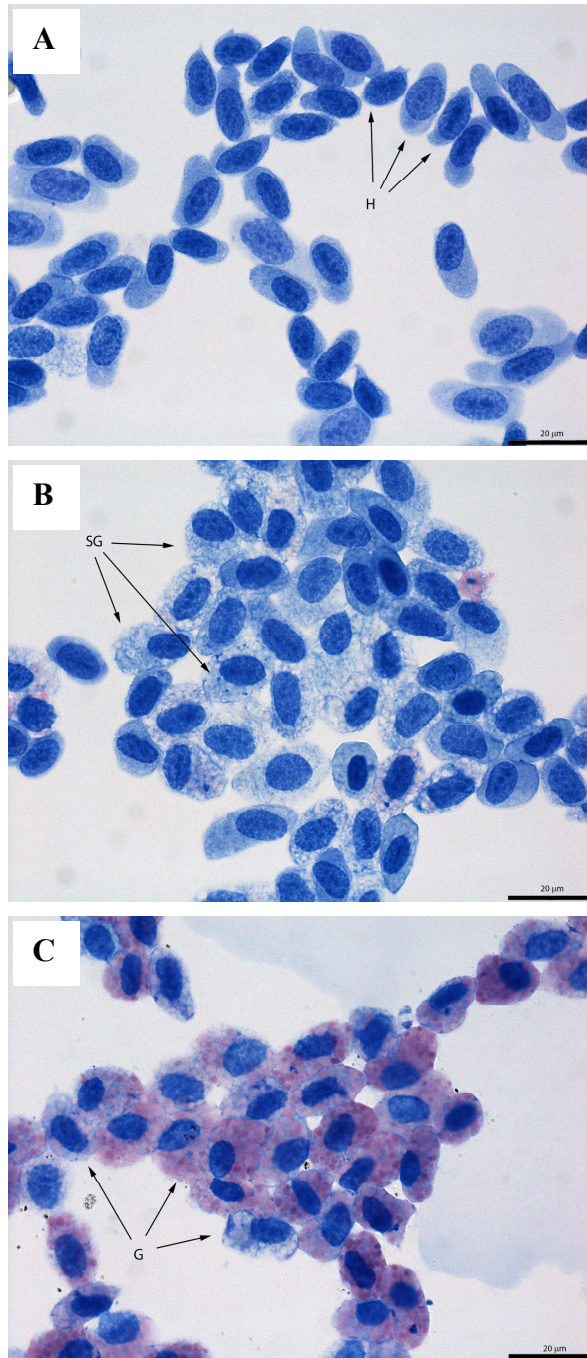


Plate 3.1. Sorted haemocytes from predefined populations stained with Leishman's-Giemsa. A) Hyaline (H), showing variation in nuclear size and staining, B) semigranular (SG), showing variation in nuclear size and staining and cytoplasmic granularity and C) granular (G), showing variation in intensity of eosinophilic staining of granules. All figures x 1000.

Table 3.1. Mean (\pm SEM) length (l) and diameter (d) for sorted hemocytes and nuclei (n = 30). Asterisks indicate significant differences ($P < 0.05$, Tukey's post-hoc test) in cell area and nucleocytoplasmic ratio between cell types.

	Hyaline				Semigranular				Granular			
	Nucleus		Cell		Nucleus		Cell		Nucleus		Cell	
	l	d	l	d	l	d	l	d	l	d	l	d
Mean (μm)	11 (0.2)	7 (0.2)	13 (0.3)	9 (0.3)	12 (0.2)	7 (0.1)	15 (0.3)	10 (0.2)	8 (0.1)	5 (0.2)	14 (0.4)	10 (0.2)
Area (μm^2)	61 (3.0)	104 (4.6)*			48 (2.2)		123 (4.6)		33 (1.3)		132 (1.0)	
Nucleocytoplasmic ratio (%)		59 (2.0)*			40 (1.7)*				25 (1.0)*			

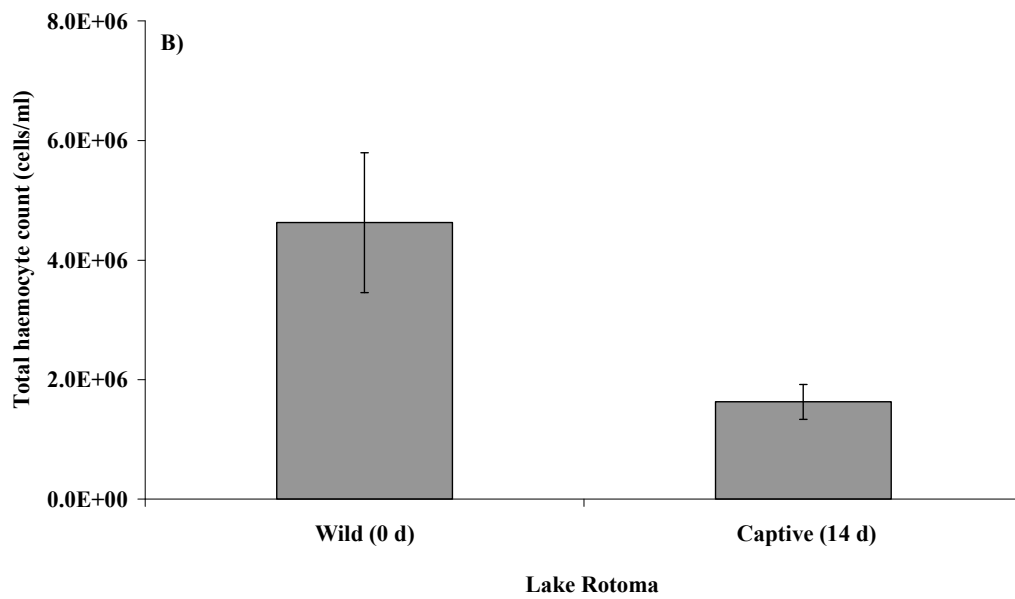
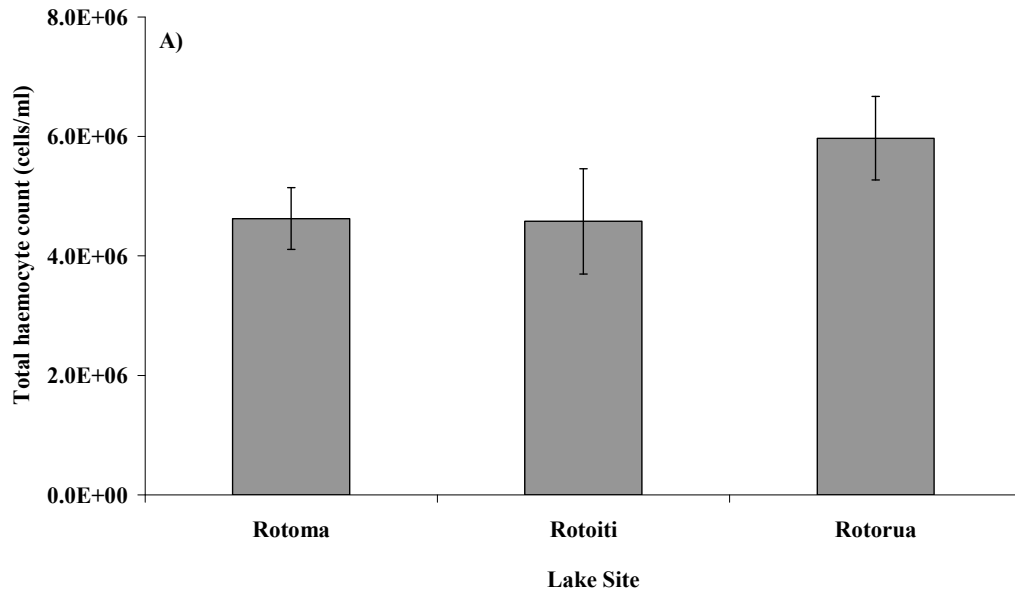


Figure 3.3. Comparative total hemocyte counts of A) wild lake specimens and B) wild vs. 14 d-captive specimens. Values are mean \pm SEM.

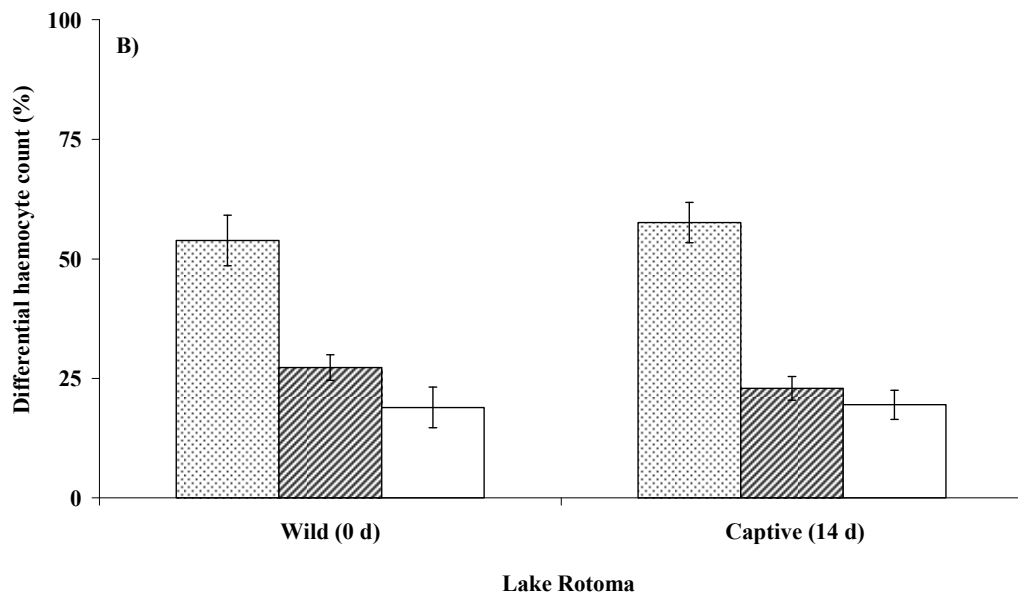
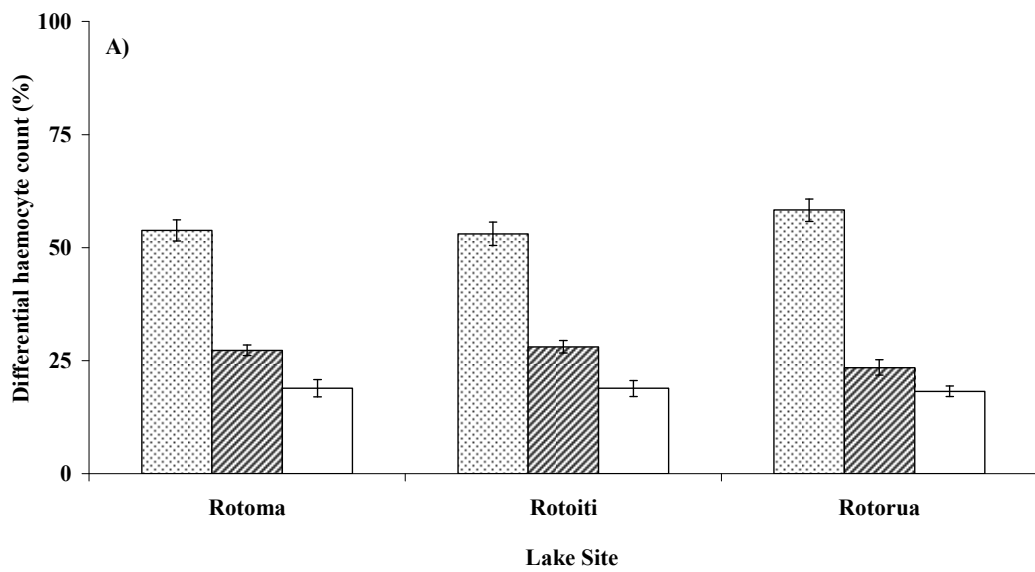


Figure 3.4. Differential hemocyte counts in A) wild lake specimens and B) wild vs. 14 d-captive specimens. Columns are hyaline (dotted), semigranular (diagonal hatch) and granular (open) cells. Values are mean \pm SEM.

3.4 Discussion

As far as we are aware, this is the first detailed investigation and characterisation of the New Zealand freshwater crayfish haemocytes. Formaldehyde was found to be the most effective sampling medium for hemolymph as cell fixation prevented clotting and enabled FC investigation and sorting of intact haemocytes. The FC facilitated a significant level of discrimination between the hemocyte types based on morphology and light scatter, which was further validated by microscopic examination. Three hemocyte populations were identified and found to be morphologically consistent with the generally accepted invertebrate hemocyte classifications. Using the FC protocol developed here, total and differential hemocyte counts were measured in several wild crayfish populations and also demonstrated a physiological effect of captivity on the circulating haemocytes.

Crustaceans are generally considered to possess three hemocyte types based on morphology; the hyaline, semigranular and granular cells (Bauchau 1981; Johansson *et al.*, 2000). Hose *et al.*, (1990) contend that morphological features alone do not accurately differentiate between the hemocyte categories in decapods, and that a combination of morphological, cytochemical and functional methods are necessary to accurately describe haemocytes. In the current study, the FC identified three hemocyte populations based on differential light scatter and fluorescence. Differentiation of sorted populations by Romanowsky (Leishman's-Giemsa) staining supported the presence of three hemocyte types and further facilitated the identification of hyaline, semigranular and granular cells in agreement with the previous crustacean hemocyte descriptions (Bauchau, 1981; Hose *et al.*, 1990; Johansson *et al.*, 2000). The agranular cells possessing a high nucleocytoplasmic ratio were consistent with the identification of crustacean hyaline cells. A characteristic decrease in nuclear size and increase in granularity between the hyaline and granular cells, and the eccentric location of nuclei in granular cells were also observed. Using probability contour plots, the FC was further capable of differentiating the granular haemocytes into two subpopulations. When examined by light microscopy, the granular subpopulations were observed to possess varying degrees of granularity and differing staining characteristics which are consistent with the identification of semi-granular and

granular cells. Our results support the suggestion of Sequeira *et al.*, (1995) that FC is more suitable for reliable identification and enumeration of crayfish haemocytes than traditional methods such as subjective morphological investigations using smears.

Although differences in THC or DHC of wild crayfish were not observed, the dramatic reduction of THC in captive animals was indicative of a definite response to capture and captivity in this species. Regardless of the possible reasons for these changes, reduced circulating hemocyte numbers represents an increased risk of infection (Le Moullac and Haffner, 2000). Moreover, the 63% reduction in THC exhibited by captive crayfish herein is similar to the observed threshold of mortality of approximately 30% of original THC in two other decapod crustacean species, the shrimps *Crangon crangon* and *Palaemon elegans* (Lorenzon *et al.*, 1999). Severe or prolonged reduction of THC may have significant implications for long-term survival in captivity and may partially explain the frequently encountered difficulties in keeping this species in the laboratory.

Changes in the circulating hemocyte numbers of crustaceans have been observed in response to a variety of stressors ranging from bacterial and fungal infection (Cheng and Chen, 2001; Jiravanichpaisal *et al.*, 2001), the bacterial endotoxin lipopolysaccharide (LPS) (Lorenzon *et al.*, 1999; Lorenzon *et al.*, 2002; Cardenas *et al.*, 2004), toxicant exposure (Smith *et al.*, 1995; Lorenzon *et al.*, 2001; Ward *et al.*, 2006) and capture stressors, such as air exposure, handling and transportation (Jussila *et al.*, 1997; Fotedar *et al.*, 2001; Tsvetnenko *et al.*, 2001; Lorenzon *et al.*, 2008). However, changes in circulating hemocyte numbers may be rapid and show near baseline recovery within hours to days. For example, Lorenzon *et al.*, (2001) demonstrated that short-term 96 h exposures to heavy metal solutions were capable of reducing THC in the shrimp *Palaemon elegans* over the first 8 h of exposure, generally returning to normal 16 h following exposure. Lorenzon *et al.* (2002) also demonstrated a similar THC decline and recovery in response to LPS injection in this species. The prolonged depression of circulating haemocytes of crayfish in the current study may be related to poor

animal recovery following capture or through continued stress related to confinement.

Alternatively, various environmental factors such as fluctuations in temperature and dissolved oxygen may also influence the crustacean immune system on a daily basis (Le Moullac *et al.*, 1998; Le Moullac and Haffner, 2000), whereas captive animals are typically subjected to constant ambient conditions. Wild animals are also likely to experience a greater natural abundance of bacteria where increased hemocyte numbers would presumably favour survival in the wild. Although many studies have documented decreased circulating haemocytes in response to challenge, other studies have reported hemocyte proliferation. Sequeira *et al.* (1996) observed 3 to 6-fold increases in circulating haemocytes in response to LPS stimulation and fungal infection in the shrimp *Penaeus japonicus*. Latex beads have also been shown to cause hemocyte proliferation in the freshwater crayfish *Astacus leptodactylus* (Giulianini *et al.*, 2007). Jussila *et al.* (2001) demonstrated an approximately 2-fold increase in THC of the rock lobster *Panulirus cygnus* 1 h post-exercise. Thus, an alternative explanation to stress-induced reductions in THC is that captive crayfish reverted to or experienced a change in baseline physiological status owing to recovery from capture, improved water quality and/or constant ambient conditions.

This study demonstrated that FC was suitable for the differentiation and enumeration of circulating haemocytes of the New Zealand freshwater crayfish and is a valuable tool for the assessment of physiological status. It is anticipated that future hematological investigations using this species will provide early indication of ecosystem stress, and thus broaden the scope of environmental impact assessments using this environmentally relevant species.

3.5 References

Baden, S. P., and D. M. Neil. 2003. Manganese accumulation by the antennule of the Norway lobster *Nephrops norvegicus* (L.) as a biomarker of hypoxic events. *Marine Environmental Research* **55**:59-71.

Bauchau, A. G. 1981. Crustaceans. Pages 385-420 in N. A. Ratcliffe, and A. F. Rowley, editors. *Invertebrate Blood Cells*. Academic Press, London.

Becton-Dickinson. 2004a. Sorting. Pages 73-114 in *BD Digital Electronics Option User's Guide*. Becton, Dickinson and Company, San Jose.

Becton-Dickinson. 2004b. Tools for data analysis. Pages 150 in *BD FACSDiVa Software Reference Manual*. Becton, Dickinson and Company, San Jose.

Bryant, C. L., J. G. Farmer, A. B. MacKenzie, A. R. Bailey-Watts, and A. Kirika. 1997. Manganese behaviour in the sediments of diverse Scottish freshwater lochs. *Limnology and Oceanography* **42**:918-929.

Cardenas, W., J. R. Dankert, and J. A. Jenkins. 2004. Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish and Shellfish Immunology* **17**:223-233.

Cardenas, W., J. A. Jenkins, and J. R. Dankert. 2000. A flow cytometric approach to the study of crustacean cellular immunity. *Journal of Invertebrate Pathology* **76**:112-119.

Cheng, W., and J.-C. Chen. 2001. Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. *Fish and Shellfish Immunology* **11**:53-63.

Fotedar, S., E. Tsvetnenko, and L. H. Evans. 2001. Effect of air exposure on the immune system of the rock lobster *Panulirus cygnus*. *Marine and Freshwater Research* **52**:1351-5.

Giulianini, P. G., M. Bierti, S. Lorenzon, S. Battistella, and E. A. Ferrero. 2007. Ultrastructural and functional characterisation of circulating haemocytes from the freshwater crayfish *Astacus leptodactylus*: Cell types and their role after *in vivo* artificial non-self challenge. *Micron* **38**:49-57.

Goedken, M., and S. De Guise. 2004. Flow cytometry as a tool to quantify oyster defence mechanisms. *Fish and Shellfish Immunology* **16**:539-552.

- Hernroth, B., S. P. Baden, K. Holm, T. Andre, and I. Soderhall. 2004. Manganese induced immune suppression of the lobster, *Nephrops norvegicus*. *Aquatic Toxicology* **70**:223-231.
- Hose, J. E., G. G. Martin, and A. S. Gerard. 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *Biological Bulletin* **178**:33-45.
- Iwanaga, S., and B. L. Lee. 2005. Recent advances in the innate immunity of invertebrates. *Journal of Biochemistry and Molecular Biology* **38**:128-150.
- Jiravanichpaisal, P., E. Bangyeekhun, K. Soderhall, and I. Soderhall. 2001. Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*. *Diseases of Aquatic Organisms* **47**:151-157.
- Jiravanichpaisal, P., B. L. Lee, and K. Soderhall. 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**:213-236.
- Johansson, M. W., P. Keyser, K. Sritunyaluksana, and K. Soderhall. 2000. Crustacean haemocytes and haematopoiesis. *Aquaculture* **191**:45-52.
- Jussila, J., J. Jago, E. Tsvetnenko, B. Dunstan, and L. H. Evans. 1997. Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. *Marine and Freshwater Research* **48**:863-867.
- Jussila, J., S. McBride, J. Jago, and L. H. Evans. 2001. Hemolymph clotting time as an indicator of stress in western rock lobster (*Panulirus cygnus* George). *Aquaculture* **199**:185-193.
- Le Moullac, G., and P. Haffner. 2000. Environmental factors affecting immune responses in crustacea. *Aquaculture* **191**:121-131.
- Le Moullac, G., and coauthors. 1998. Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. *Fish and Shellfish Immunology* **8**:621-629.

- Lorenzon, S., S. De Guarrini, V. J. Smith, and E. A. Ferrero. 1999. Effects of LPS injection on circulating haemocytes in crustaceans *in vivo*. *Fish and Shellfish Immunology* **9**:31-50.
- Lorenzon, S., M. Francese, V. J. Smith, and E. A. Ferrero. 2001. Heavy metals affect the circulating haemocyte number in the shrimp *Palaemon elegans*. *Fish and Shellfish Immunology* **11**:459-472.
- Lorenzon, S., P. G. Giulianini, S. Libralato, M. Martinis, and E. A. Ferrero. 2008. Stress effect of two different transport systems of the physiological profiles of the crab *Cancer pagurus*. *Aquaculture* **278**:156-163.
- Lorenzon, S., P. Pasqual, and E. A. Ferrero. 2002. Different bacterial lipopolysaccharides as toxicants and stressors in the shrimp *Palaemon elegans*. *Fish and Shellfish Immunology* **13**:27-45.
- Owens, L., and A. O'Neill. 1997. Use of a clinical flow cytometer for differential counts of prawn *Penaeus monodon* haemocytes. *Diseases of Aquatic Organisms* **31**:147-153.
- Sequeira, T., D. Tavares, and M. Arala-Chaves. 1996. Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*. *Developmental and Comparative Immunology* **20**:97-104.
- Sequeira, T., M. Vilanova, A. Lobo-da-Cunha, L. Baldaia, and M. Arala-Chaves. 1995. Flow cytometric analysis of molt-related changes in hemocyte type in male and female *Penaeus japonicus*. *Biological Bulletin* **189**:376-380.
- Shapiro, H. M. 2003. *Practical Flow Cytometry*. John Wiley and Sons, Inc.
- Smith, V. J. 1991. Invertebrate immunology: phylogenetic, ecotoxicological and biomedical implications. *Comparative Haematology International* **1**:61-76.
- Smith, V. J., R. J. Swindlehurst, P. A. Johnston, and A. D. Vethaak. 1995. Disturbance of host defence capability in the common shrimp, *Crangon crangon*, by exposure to harbour dredge spoils. *Aquatic Toxicology* **32**:43-58.

Soderhall, K., and V. J. Smith. 1983. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Developmental and Comparative Immunology* **7**:229-239.

Sokal, R. R., and F. J. Rohlf. 1973. *Introduction to biostatistics*. W. H. Freeman and Company, San Francisco, CA, USA.

Tankere, S. P. C., P. J. Statham, and N. B. Price. 2000. Biogeochemical cycling of Mn and Fe in area affected by eutrophication: the Adriatic Sea. *Estuarine, Coastal and Shelf Science* **51**:491-506.

Theopold, U., O. Schmidt, K. Soderhall, and M. S. Dushay. 2004. Coagulation in arthropods: defence, wound closure and healing. *Trends in Immunology* **25**:289-294.

Travers, M.-A., and coauthors. 2008. Morphologic, cytometric and functional characterisation of abalone (*Haliotis tuberculata*) haemocytes. *Fish and Shellfish Immunology* **24**:400-411.

Tsvetnenko, E., S. Fotedar, and L. Evans. 2001. Antibacterial activity in the haemolymph of western rock lobster, *Panulirus cygnus*. *Marine and Freshwater Research* **52**:1407-1412.

Ward, R. J. S., C. R. McCrohan, and K. N. White. 2006. Influence of aqueous aluminium on the immune system of the freshwater crayfish *Pacifasticus leniusculus*. *Aquatic Toxicology* **77**:222-228.

Xue, Q.-G., T. Renault, and S. Chilmonczyk. 2001. Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. *Fish and Shellfish Immunology* **11**:557-567.

Yip, E. C. H., and J. T. Y. Wong. 2002. Fluorescence activated cell-sorting of haemocytes in Penaeid prawns. *Aquaculture* **204**:25-31.

Chapter Four

General Conclusions and Recommendations

4.1 Conclusions

The aim of this study was to develop methodologies for assessing blood and haemolymph of freshwater fish and crayfish, respectively, which may be employed in determining species-specific physiological responses to environmental stressors.

Flow cytometry was found to be a useful technique in the enumeration and differentiation of the peripheral blood cells of rainbow trout. Total RBC and partial differentiation of leukocytes in whole blood could be determined using DiOC₆(3), a fluorescent cyanine dye, but inconsistent response of thrombocytes to fluorescent staining between individuals limited complete differentiation. This was resolved using a fluorescent-labelled trout thrombocyte mAb in combination with DiOC₆(3), which facilitated total RBC and WBC that were verified to be comparable with manual counting methods. This approach also resolved the morphological ambiguity between thrombocytes and lymphocytes that evidently compromised accurate manual DWBC. Rainbow trout was chosen as a physiologically well-understood model to develop FC haematological methods for New Zealand native fishes, but time restriction limited this study to that of trout alone. However, on the basis of the results achieved, extension of the methodology to include native species seemed feasible.

Flow cytometry was also found to be useful in the characterisation and enumeration of the haemocytes of the New Zealand freshwater crayfish, for which very little physiological information appeared to exist. The haemolymph could not be effectively stabilised by the anticoagulant formulations evaluated, necessitating sampling with formaldehyde as a fixative to preserve haemocyte morphology and cellular integrity. Three haemocyte types were characterised on the basis of light scattering properties using FC, and Romanowsky staining of isolates to examine morphology. Haemocytes were found to morphologically conform to the established crustacean haemocyte classification system descriptions of hyaline, semigranular and granular cells. Despite incomplete resolution of the three populations on FC scatter plots, likely due to the effects of fixation (Givan, 2000), use of the “20% probability” instrument software function facilitated consistent differentiation of gated populations between specimens.

During the course of the study, the developed FC protocols for trout and crayfish were successfully applied by our researchers in the unrelated assessment of wild populations (Landman *et al.*, 2007; Landman and Ling, 2008; Landman *et al.*, 2008). Bleackley (2008) used a modification of the trout FC methodology in the evaluation of RBC and WBC of common bully (*Gobiomorphus cotidianus*) in Bay of Plenty habitats.

Flow cytometry is a versatile, rapid and more accurate alternative to manual techniques used for the enumeration, characterisation and differentiation of blood cells and haemocytes of fish and crustacea in developing methodologies for examining physiological status.

4.2 Recommendations

In developing FC methods for other freshwater fish species, it is recommended that the anticoagulant and diluent compatibility studies performed on trout be similarly evaluated. The microscopic appearance of the blood cells in smears of trout and those of common bully (Bleackley, 2008) were observed to be morphologically different, and it should not be assumed, for example, that the optimal anticoagulant or dosage for trout will be suitable for other species. Hattingh (1974) observed variant dosage effects of anticoagulants, such as lysis and changes in Hct, between multiple species.

3,3'-Dihexyloxycarbocyanine iodide was used as a fluorescent dye in this study due to its extensive and well-described use in determining membrane potential in human medicine (Shapiro, 2003), and its previous use in FC studies of carp (Inoue *et al.*, 2002). DiOC₆(3) is, however, only one of several cyanine dyes that have been used in FC studies. Uchiyama *et al.* (2005) demonstrated the use of 3,3'-dipentyloxycarbocyanine (DiOC₅(3)) to resolve thrombocytes and lymphocytes in quail blood; resolution was not achieved using DiOC₆(3). It is recommended that additional cyanine dyes be evaluated on rainbow trout blood, with a view to achieving resolution of leukocytes without the need for mAb. Acridine Orange has been used in the analysis of cyprinid and salmonid blood (Morgan *et al.*, 1993; Inoue *et al.*, 2002), but without clear resolution of

thrombocytes and leukocytes. Its use has also been discouraged on the BD FACSVantage™ SE instrument by the supplier, due to its affinity to and slow release from fluidics system surfaces. For *in vitro* studies requiring high purity sorting, such as those relating to specific immunity, the use of mAb is recommended. An extensive range of mAb to trout leukocytes have been raised (Hoeger *et al.*, 2004).

A limitation of this study was the lack of a suitable anticoagulant for the isolation of vital freshwater crayfish haemocytes. The use of fixative precludes the use of haemocytes for *in vitro* procedures that may help to evaluate the physiology of these animals. Flow cytometry has been used in evaluating crustacean immunity *in vitro* by measuring haemocyte responses, in terms of changes in scatter properties and cell viability, to fungal cell walls and lipopolysaccharide (Cardenas *et al.*, 2000; Cardenas *et al.*, 2004). *In vivo* determination of sub-lethal effects of immunological stimuli may, however, be evaluated with the current methodology using fixed cells. Similarly, using the current methodology, the immediate opportunity exists to evaluate the comparative haemocyte properties of both the Northern and Southern (*Paranephrops zealandicus*) New Zealand freshwater crayfish, to determine if these species are physiologically as well as morphologically distinct. The use of electron microscopic techniques in the characterisation of intra-cellular structure of haemocytes (Johansson *et al.*, 2000), isolated by FC, may facilitate a better understanding of the function and life cycle of haemocytes in these species, based on the wealth of fundamental knowledge of crustacean species currently available.

4.3 References

Bleackley NA. 2008. Biology of common bully (*Gobiomorphus cotidianus*) populations in the Tarawera and Rangitaiki rivers: reproductive isolation by inland distance or effluent discharges? Unpublished MSc thesis, The University of Waikato, Hamilton, New Zealand.

Cardenas W, Dankert JR, Jenkins JA. 2004. Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish and Shellfish Immunology* **17**:223-233.

Cardenas W, Jenkins JA, Dankert JR. 2000. A flow cytometric approach to the study of crustacean cellular immunity. *Journal of Invertebrate Pathology* **76**:112-119.

Givan AL. 2000. Critical aspects of staining for flow cytometry. In: Diamond R, De Maggio S, editors. *In Living Colour: Protocols in Flow Cytometry and Cell Sorting*. Berlin: Springer. p 142-164.

Hoeger B, Koellner B, Kotterba G, Van den Heuvel MR, Hitzfeld B, Dietrich DR. 2004. Influence of chronic exposure to treated sewage effluent on the distribution of white blood cell populations in rainbow trout (*Oncorhynchus mykiss*) spleen. *Toxicological Sciences* **82**:97-105.

Inoue T, Moritomo T, Tamura Y, Mamiya S, Fujino H, Nakanishi T. 2002. A new method for fish leukocyte counting and partial differentiation by flow cytometry. *Fish and Shellfish Immunology* **13**:379-390.

Johansson MW, Keyser P, Sritunyalucksana K, Soderhall K. 2000. Crustacean haemocytes and haematopoiesis. *Aquaculture* **191**:45-52.

Landman MJ, Brijs J, Glover CN, Ling N. 2007. *Lake Okareka and Tikitapu fish health monitoring 2007*. Contract Report to Environment Bay of Plenty. 34 p.

Landman MJ, Ling N. 2008. *Lake Okaro and Rerewhakaaitu fish health monitoring: Okaro Z2G1 trial*. Contract report to Environment Bay of Plenty. Rotorua: Scion. 50 p.

Landman MJ, Ling N, Taylor SC. 2008. Fish community surveys and biomonitoring in selected Rotorua streams. Contract report to Environment Bay of Plenty. Rotorua: Scion. 33 p.

Morgan JAW, Pottinger TG, Rippon P. 1993. Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *Journal of Fish Biology* **42**:131-141.

Shapiro HM. 2003. Parameters and probes. *Practical Flow Cytometry*. 4th ed. Hoboken: John Wiley & Sons, Inc. p 273-409.

*City life was gettin' us down
So we spent a weekend outta town
Pitched our tent on a patch of ground
Down by the river
Lit a fire and drank some wine
You put your jeans on top of mine
I said "Come in, the water's fine"
Down by the river*

*Didn't feel too good all night
So we took a walk in the morning light
And came across the strangest sight
Down by the river
Silver fish lay on its side
It was washed up by the early tide
I wonder how it died
Down by the river*

*Doctor put us both to bed
He dosed us up and he shook his head
"Only foolish people go" he said
"Down by the river"
"Why do willows weep?" said he
"Because they're dying gradually
From the waste from the factories
Down by the river"*

*In time the riverbanks will die
The reeds will wilt, and the ducks won't fly
There'll be a tear in the otter's eye
Down by the river
The banks will soon be black and dead
And where the otter raised its head
Will be a clean, white skull instead
Down by the river*

© 1972 EMI/April Music. Used with personal permission from Albert Hammond.