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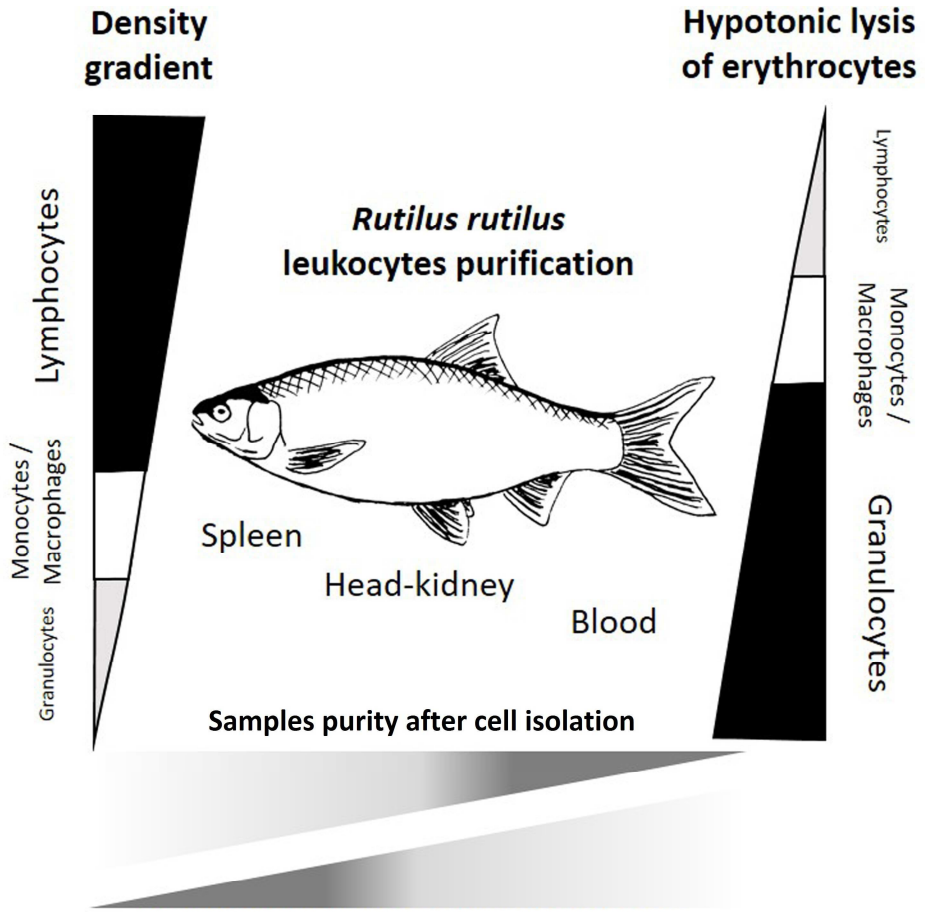

Variability in leukocytes subtypes distributions before and after cell incubation

Procedures for leukocytes isolation from lymphoid tissues and consequences on immune endpoints used to evaluate fish immune status: a case study on roach (*Rutilus rutilus*)

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Abbreviations: DCFH-DA: 2',7'-dichlorofluorescin diacetate; DG: density gradient; FCS: foetal calf serum; HL: hypotonic lysis, P/S: Penicillin/Streptomycin; PBS: Phosphate Buffer Saline ; PI: propidium iodide; ROS: reactive oxygen species; SI: stimulation index.

Abstract:

The effects of two protocols (density gradient *versus* hypotonic lysis) used for leukocyte isolation from three major lymphoid tissue of fish (head-kidney, spleen and blood) were examined on some cell functional activities (tissue leucocytes distributions, phagocytosis, basal and burst oxidative activities) classically used to estimate the fish immune status. Experiments were conducted on roach (*Rutilus rutilus*), a cyprinid fish model often studied in different eco-physiological contexts (aquaculture, ecotoxicology…). All of immune endpoints were assessed either immediately after cell isolation or after a 12h of incubation in order to observe if a post-isolation incubation may influence the leukocytes activities.

Compared to the density gradient, hypotonic lysis is associated with granulocytes enrichments of cell suspensions. This is particularly true for leukocyte suspensions isolated from head kidney where granulocytes are naturally abundant. However, important variabilities in leucocyte distributions were observed in head kidney and spleen cells samples obtained by the use of hypotonic lysis for two incubation conditions used (no incubation or 12 h of incubation at 4 ° C). The density gradient protocol leads to a transitory increase in basal ROS production in spleen lymphocytes and macrophages The blood leukocytes isolated by this same method exhibit high basal oxidative activities after 12 h of incubation at 4 ° C and for the three leukocyte types (lymphocytes, monocytes and granulocytes). The hypotonic lysis is associated with an increase in PMA-induced ROS production especially in head kidney leukocytes. The increases in cell oxidative activities are consistent with increases in granulocyte proportions observed in leukocyte suspensions obtained by hypotonic lysis. Finally, the two protocols have no effect on leucocyte mortality and phagocytic activity.

Within limits of our experimental conditions, the spleen is the organ whose leucocyte oxidative activities (stimulated or not) are only slightly influenced by the methods used for leukocyte isolation. This is also the case for the anterior kidney, but for this tissue, it is necessary to incubate the isolated cells for 12 h at 4 ° C before functional analyses.

Each of the two methodologies used has advantages and disadvantages. The hypotonic lysis allows to isolate a greater variety of leukocytes types whereas the density gradient used ensures a better stability of cells distributions over time. However, for the same fish species and for the same tissue, the method used to isolate leukocytes influences results and must be taken into consideration during acquired data analysis for evaluation of fish immune status.

1. Introduction

Immunity has an integrative and central role in physiological regulations of organisms. Even if the term of immunocompetence was not well defined yet, it represents an average value of the immunity of an organism exposed to multiple parasites and stressors [1]. It is important but not easy to evaluate immunocompetence of organisms in their own environment. As all the vertebrates, the fish immune system is a complex, multifaceted and coordinated system working to destroy pathogens and also to maintain organism integrity. Thus, it must be emphasized that a measure of a single immune endpoints is not sufficient to capture the complexity of an immune response and to estimate the immune status of an organism.

As observed in all vertebrates, fish immune response implicate various cell types. These cellular defence systems corresponds to phagocytic cells similar to macrophages, neutrophils and natural killer (NK) cells, as well as T and B lymphocytes [2–4]. The main leucocyte responses often studied in fish immune ecophysiology are represented by intracellular reactive oxygens species production associated with pathogens phagocytosis [5–8] In fish immune ecophysiology, other cellular parameters as for instance apoptosis or lysosomal activity may complete these two main functions by the use of flow cytometry techniques [9–13]. Flow cytometry provides multi-parametric data that can be used in simultaneously analysis of numerous cell properties. By this technique, it is also possible to differentiate lymphocytes, monocytes/macrophages and granulocytes and to link responses of observed cellular activities with the different types of cells present in the tissue sample. Such analysis can be perform on many different lymphoid tissues (spleen, head-kidney, blood). All of these multi-parametric

and multi-tissues data help to improve the estimation of the immune status of fish in relation to the state of their living environment.

To perform such observations on immune cells, all tissue samples must be in a single cell suspension. Indeed, before cytometric measurements of functional cellular activities, the major initial methodological step is to collect the leukocytes from the different lymphoid tissues. The erythrocyte presence in biological samples may create another difficulty especially in case of species as teleost fish which possess nucleated red blood cells. Samples contaminations by erythrocytes may compromise cytometric analysis.

The dual necessity to obtain single cell suspensions of leukocytes with no erythrocytes contaminations constitutes the actual main methodological constraint to evaluate fish immune responses.

Among the different techniques, hypertonic lysis and density gradient are the two main way used in literature to separate leukocytes from erythrocytes. Many isolation methods of vertebrates leucocytes exists and were extensively studied as hypertonic shock using ammonium chloride [14], positive selection [15], self-generating gradient [16,17], cryopreservation [18], adherence and agglutination [19] and density gradient. The use of Ficoll® or Percoll® to constitute density gradients is one of the most common isolation procedure for vertebrate leucocytes [20]. However, Ficoll® gradients may bring out some alterations in cell responses and cell surface markers in addition to cell morphological changes [21].

Purification of mammalian leukocytes by erythrocyte lysis with hypertonic ammonium chloride was usually used and is efficient in case of lysis of mammalian non-nucleated erythrocytes. However, removing erythrocytes from fish leukocyte suspensions by the use of ammonium chloride was not successful as the presence of nuclei in teleost erythrocytes may preclude lysis [22]. In 2001, another technique corresponding to an hypotonic lysis of teleost erythrocytes in peripheral blood and pronephrotic leukocytes samples was proposed by

Crippen *et al.* (2001) and was successful used in different studies where fish leukocytes immune responses need to be evaluated [24–27].

But whatever the methodology used in fish immunological investigations (density gradient, hypotonic lysis), it is important to estimate its influence on acquired data in terms of leukocytes activities and distributions.

Hence, we compared here the effects of two protocols (density gradient *versus* hypotonic lysis) for leukocyte isolation from three major lymphoid tissue of fish (head-kidney, spleen and blood) on some cell functional activities (tissue leucocytes distributions, phagocytosis, basal and burst oxidative activities) classically used to estimate the fish immune status. Experiments were conducted on roach (*Rutilus rutilus*), a cyprinid fish model often studied in different eco-physiological contexts (aquaculture, ecotoxicology…). All of these immune endpoints were assessed either immediately after cell isolation or after a 12h of incubation in order to observe if a post-isolation incubation may influence the leukocytes activities [28].

2. Materials and methods:

2.1. Chemicals and reagents:

Culture media Leibovitz L15, penicillin and streptomycin (P/S), heparin, propidium iodide (PI), 2',7'-dichlorofluorescin Diacetate (DCFH-DA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. Ficoll® (Ficoll®-Paque PLUS) was obtained from GE Healthcare, fetal calf serum (FCS) from Biochrom AG and fluorescent latex beads from Biovalley. Phosphate Buffer Saline (PBS 10X) was prepared as described by Dulbecco and Vogt (1954).

2.2. Fish:

Adult roach (*Rutilus rutilus*: 12±0.5 cm length and 12.8±1.25 g weight; Age: 2+) were purchased from a local fish producer (Saint-Mard-sur-le-Mont, France) and acclimated in laboratory in 400 L tanks with continuously filtered water for a period of 3 weeks before

experiments. Fish were fed every 2 days with frozen red mosquito larvae with regular physical-chemical conditions record and maintain (pH: 7.19 ± 0.26; Conductivity: 237 ± 28 µS; Oxygen saturation: 9.67 ± 0.46 mg/L; Temperature: 10.4 ± 0.45 °C and photoperiod of 12 hours dark/light). Experiments were conducted in April corresponding to reproduction period in the roach life cycle [30]. Protocols of fish and experimentation were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 63/2010/EU.

2.3. Lymphoid tissues collection:

Peripheral blood from ten fish was sampled from caudal vein with 1mL syringe containing 50 µL of heparin (500 UI/mL). Each sample was then diluted in L15 medium containing 500 UI/mL and 500 µg/mL of P/S respectively. Fish were then immediately sacrificed by spinal dislocation. Head-kidney and spleen were aseptically removed, passed through a 100 µm sterile nylon mesh and homogenised in 2 mL of L15 P/S containing heparin (10 UI/mL).

All samples were then separated into two equal parts. Leukocytes contained in the samples were then purified by two different methods as described as follow.

2.4. Methods used for leukocytes isolation:

2.4.1. Density gradient:

For each sample (head kidney, spleen or blood), 1mL of cell suspension was diluted in 8mL L15 P/S containing heparin (10 UI/mL) and layered onto 3 mL of Ficoll® (density : 1.077 g/mL) and centrifuged at 400 *g* for 30 minutes at 4°C. Leukocytes were collected at the culture medium-Ficoll® interface and washed once in L15 P/S (centrifugation 400 *g* for 10 minutes at 4°C). Supernatant was discarded and cells were resuspended in 1 mL of L15 P/S.

2.4.2. Hypotonic lysis of erythrocytes:

The protocol used was modified from Crippen *et al*. (2001). Nine mL of cold sterile distilled water was added to 1 mL of cell suspension. Tubes were mixed softly for 40 seconds in order

to lyse the most of erythrocytes contained in samples. This time was valid for the three tissues (blood, spleen and head kidney). One mL of PBS 10X was immediately added to adjust the sample osmolality. Suspensions were then centrifuged at 400 *g* for 10 minutes at 4°C.

For head-kidney samples, ~~viscous pellet was removed,~~ supernatant was discarded and ~~cell pellet was resuspended in~~ 1mL L15 penicillin/streptomycin with 5% of heat inactivated fetal calf serum was added. The viscous pellet with cells was gently pumped many times with a 5 mL pipet to release the most of cells. Then the viscous mass was discarded. For peripheral blood and spleen samples, viscous mass with cells was collected from the top of erythrocytes debris ~~leucocytes pellets~~ and transferred into a new tube containing 1 mL L15 penicillin/streptomycin with 5% of heat inactivated fetal calf serum. Afterwards, cells were separated from the viscous pellet as done for head-kidney samples. ~~Collected leukocytes were diluted in 1mL L15 P/S containing 5% of heat inactivated fetal calf serum~~.

All the samples were analysed immediately after the leukocyte isolation procedure and also after a 12 h of incubation at 4 ° C. After ~~12h of~~ incubation, cells were washed once by centrifugation (400 *g*, 10 minutes at 4°C) and suspended in 1 mL of L15 P/S.

2.5. Leukocytes parameters

2.5.1. Leukocytes distributions and concentrations:

~~*2.5.1 Leukocytes distribution:*~~

~~Following isolation, all samples were diluted to 1:10 in phosphate buffer saline for cytogram examination and comparison.~~

Representative cytograms obtained on leukocytes suspension isolated from head-kidney are represented in figure 1. Gate P1 was related to leukocytes (Figure 1 A and D). Gate P2 was done to exclude doublets by the use of FSC-H/FSC-A cytogram (Figures 1 B and E). Gate P3 was drawn to eliminate impurities as cell debris (Figures 1 C and F).

A total of 10000 events were recorded in P1 (Leukocytes) after exclusion of doublets. Data were analyzed with the Accuri™ C6 software. For leukocytes distributions, cell subtypes

were separated according to their forward and side scatter parameters (FSC-A versus SSC-A dotplots) (Figure 2.). For abbreviations of cell names, Ly corresponded to lymphocytes while this gated subpopulation contained also thrombocytes [10,31,32].

After doublets exclusion (Figure 1; P2 gate), total events recorded in leukocytes gate (P1) from 100 µL of diluted tissue samples in PBS allowed to establish leukocyte concentration in cell per mL.

2.5.2. Samples purity:

The amount of sample purity was obtained by subtracting the percentage of events of the impurities gate (P3) from the whole analysed volume of sample (100%) when events in leukocytes gate reached 10000 with doublets exclusion.

2.6. Cellular functional tests:

For each sample, $2x10^5$ cell/mL were deposited in 96-U bottom wells microplates for functional cellular parameters analysis by flow cytometry. All cytometric measurements were carried out with an Accuri™ C6 flow cytometer (Becton Dickinson). ~~For each leukocyte sample, 10,000 events were recorded and leucocytes were distinguished according to their morphological parameters, forward scatter (FSC-A) for particle size and side scatter (SSC-A) for internal complexity.~~

2.6.1 Leukocytes mortality:

Cell mortality was evaluated using propidium iodide (PI) probe at 1µg/mL. Analysis was carried by flow cytometry using 488nm excitation laser and measuring fluorescence in associated channel through 585/15nm filter. Dead cells with injured membrane were positive to the red fluorescence-emitting probe bound to DNA. Results were expressed as mortality percentages corresponding to PI-positive cells (Fig.3. A and B).

2.6.2. Phagocytosis assay:

Leukocytes were incubated with yellow-green fluorescent latex beads (Fluoresbrite®, Polyscience; 2 µm diameter) for 18h at 16°C with a 1/100 leukocyte-beads ratio prior cytometric analysis measuring fluorescence in FL-1 channel (533/30) (Fig 4).

While adherence of foreign particles is the first step of the phagocytosis mechanism, the phagocytic activity measured corresponds to percentages of cells ingesting and/or adhering three beads and more. The mean number of ingested beads per phagocytic cells was calculated by dividing the mean fluorescence of events corresponding to three beads and more by the mean fluorescence of events corresponding to only one bead (Fig 4) [33].

2.6.3. Oxidative activity assay:

The leukocyte oxidative activity was quantified using flow cytometry to measure intracellular hydrogen peroxide production following activation or not with phorbol 12-myristate 13-acetate (PMA). The fluorescence levels of DCFH was measured in unstimulated and PMA-stimulated cells.~~were determined after 30 minof~~

Cells were incubated with 2′-7′dichlorofluorescin diacetate (DCFH-DA, 500nM, in obscurity at room temperature) for 15 minutes. Phorbol Myristate acetate (PMA, 2µg.mL^{-1}) was then added or not (control) to cell suspensions and cells were incubated for 30 minutes in obscurity and at room temperature. Basal intracellular ROS production corresponds to the mean fluorescence of DCFH (in FL1) measured ~~without any stimulation~~ in control whereas activated intracellular ROS production was the mean fluorescence of DCFH (in FL1) measured in cell after 30 minutes of incubation with PMA. For activated ROS production, ~~the~~ results were expressed in stimulation index as the ratio between the mean fluorescence measured in stimulated cells (DCFH-DA + PMA) and the basal mean fluorescence of control (DCFH-DA only) (Fig 5).

2.7. Statistical analysis:

Since data sets did not have a normal distribution and/or homogeneity of variance, all biological data were represented in boxplots with 25th and 75th percentiles over and below the median line within the box (N=10 different observations). Non-parametric Mann-Whitney U test was used to compare data obtained from each cell treatment ($p<0.05$ is considered statistically significant).

As all data were obtained on the same cell populations, correlations between leukocytes distributions and cell immune responses were calculated with the non-parametric Spearman correlations test. Values of correlation coefficients (ρ values) were significant at $p<0.05$. After Fisher's data transformation, differences between ρ values obtained from different cell treatment (two different incubation times or two protocols for leukocyte isolation) were evaluated with Z-test of significance ($p<0.05$).

All data were statistically analysed with Statistica software (StatSoft, Inc. (2011). STATISTICA (data analysis software system, version 10. www.statsoft.com)).

3. Results:

3.1. Leukocyte cytograms profiles:

Cytograms of isolated leukocytes show that ~~Head-kidney profiles show the highest cell contents and were then used to gate dot plots and delimit sub-populations areas on cytograms (Fig.1 -D). three leucocytes sub-populations (lymphocytes, monocytes/macrophages, granulocytes) and especially~~ granulocytes were more visible on cytograms of cell suspensions obtained by hypotonic lysis (HL) in comparison with cytograms of leukocytes isolated by density gradient centrifugation on both times of analysis (no incubation and after 12 hours of incubation) (Fig 6) ~~(Fig.1)~~. Head-kidney isolated leukocytes denoted also higher content of granulocytes. ~~This observation was also valid for spleen and blood leukocytes cytograms (Fig. 1).~~

3.2. Leucocytes concentrations:

In this study, each sample was treated for leukocytes collection using the two methods simultaneously and side by side. The median of yield obtained from 10 fish organs does not reflect the average leukocytes number in a whole collected organ (Table1).

Head-kidney leukocytes concentrations obtained with HL protocol is visually higher than cell concentrations provided after density gradient centrifugation of cells ($14x10^6$ cell/mL for HL than $8.93x10^6$ cell/mL for DG) (Table 1). However, no significant difference appears between these two methods as the min–max values were in the same range (DG: [$4.69x10^6$–$20.4x10^6$] and HL: [$7.83x10^6$– $24.1x10^6$]) (Table 1). This high variability in results may explain this non-statistical difference despite of difference observed for median cell concentrations obtained. For the two other tissues (blood and spleen), no statistical difference was observed for median leukocytes concentrations between the two protocols used (Table 1).

3.3. Comparison of samples purity between isolation methods:

Cytograms were also exploited as tool for evaluation of samples purity following isolation. Small debris at the origin of cytograms axis were excluded. Leukocytes dots and thrombocytes were gated ~~together~~ and the rest was assigned as impurities: debris and cell ghosts. Thrombocytes were included in measurements as there are indistinguishable from lymphocytes. ~~methods resulted in their collection with leukocytes. It is important to note that for leukocyte distribution measurements, thrombocytes are not included in analysis. Only lymphocytes, monocytes/macrophages and granulocytes are taken into account.~~

Cell isolation by DG led to significant higher purity levels in leukocytes for obtained cell suspension in comparison with cell suspensions where leukocytes were isolated by HL (Table 2). This observation was valuable for the three tissues used (spleen: $p=0.0051$; head–kidney:

277 *p=0.00002*; blood: *p=0.0038*). The difference is even more important for blood tissue (97.7%
278 with DG and 89.2% with HL protocols) (Table 2).

279 *3.4. Leukocytes sub-populations distributions:*

280 HL protocol was associated with cell suspensions containing more granulocytes than those
281 obtained after DG protocol whatever the post-isolation incubation time used (Fig. 7). The
282 greater difference was observed for head–kidney isolated leukocytes particularly when
283 leukocytes suspension were incubated for 12 h after isolation (*p=0.00007* for lymphocytes,
284 *p=0.023* for monocytes/macrophages and *p=0.00002* for granulocytes) (Fig. 7). For cell
285 isolated from spleen, the most notable difference between the two protocols were observed for
286 leukocytes distributions evaluated immediately after isolating procedure (*p=0.0004* for
287 lymphocytes, *p=0.023* for monocytes/macrophages, *p=0.0038* for granulocytes) (Fig. 3).
288 After 12 h of incubation, only percentages of granulocytes were significantly higher in cell
289 suspensions obtained after HL than those obtained after DG (*p=0.00001* for granulocytes)
290 (Fig. 7). The HL method also brought significant difference in blood tissue with more
291 collected lymphocytes and granulocytes especially after purification (*p=0.011* for
292 lymphocytes and *p= 0.014* for granulocytes) and with more collected granulocytes after 12h
293 of cell incubation (*p=0.022*) (Fig. 7).

294 It is important to note that density gradient in terms of leukocytes distribution has more
295 stability. In fact, leukocytes show near percentage of composition right after isolation or after
296 12h incubation in all organs with slightly more stability in spleen (No incubation :
297 lymphocytes 82.34%; monocytes/macrophages: 13.14%; granulocytes: 2.24% - 12 hours of
298 incubation: lymphocytes 87.09%; monocytes/macrophages: 11.91%; granulocytes: 0.92%)
299 than head-kidney (No incubation : lymphocytes 65.29%; monocytes/macrophages: 12.33%;
300 granulocytes: 19.58% - 12 hours of incubation: lymphocytes 52.73%;
301 monocytes/macrophages: 15.54%; granulocytes: 26.58%) or blood (No incubation :

lymphocytes 87.66%; monocytes: 9.74%; granulocytes: 1.45% - 12 hours of incubation: lymphocytes 76.42%; monocytes: 17.72%; granulocytes: 3.83%).

3.5. Leukocytes mortality:

For splenocytes, a significant greater variability on cells mortality rates is noticeable immediately after cell isolation, for total leukocyte populations when using HL compared to DG protocol (DG: 3.08%; HL: 8.71%; *p=0.035*) (Fig.8.A). Nevertheless, amounts of mortality are in the same range for total leukocytes and the various subtypes after 12h of cell incubation. Mortality measured in total head kidney-isolated leukocytes immediately after their isolation was significantly higher after DG than after HL protocol (3.99% and 2.64% respectively; *p=0.018*) (Fig.8.B). ~~Any~~ No other difference was observed for head-kidney cells. Freshly isolated blood leukocytes had significant high mortality with HL compared to DG (4.81% and 1.36%; *p=0.002*). This cell death concerned lymphocytes sub-types (0.59% for DG and 1.87% for HL; *p=0.043*) (Fig.8.C). A higher variability in monocytes/macrophages mortality was observed following the isolating protocol (Fig.8.C). ~~Any~~No other differences in cell mortality appeared after 12h of cell incubation in all cell types.

3.6. Basal oxidative activity:

Just after the cell isolation phase, splenocytes (particularly lymphocytes and monocytes/macrophages), isolated with DG had significant higher basal ROS production in comparison with cell isolated by HL method (*p=0.0003* and *p=0.0002* respectively) (Fig. 5A). After 12h of post-isolation incubation, there ~~were~~ were no~~any more~~ differences in basal oxidative activity between the two cell isolating procedures (Fig.9.A).We noticed that basal oxidative activity tends to be greater for granulocytes compared to the two other cell types. Whatever procedure used to isolate head kidney leukocytes, their basal ROS production did not differ between the different cell populations immediately after cell isolation and also after

12h incubation (Fig.9.B). However, we can notice a higher interindividual variability in basal oxidative activity of head-kidney leukocytes just after their isolation by DG (Fig.9.B). After 12h of incubation, this variability in basal ROS production disappeared for head kidney cells isolated by DG and appeared with head kidney cells isolated by HL. All blood leukocytes subtypes incubated for 12h after isolating procedure had higher basal oxidative activity when they were isolated by the use of DG in comparison with same cell types isolated by HL (*p=0.00008* for lymphocytes, *p=0.00008* for monocytes/macrophages, *p=0.011* for granulocytes) (Fig.9.C).

3.7. PMA-stimulated oxidative activity:

Granulocytes of DG-purified spleen leukocytes show higher SI comparatively to HL-purified ones after purification but with no statistically significant difference (Stimulation index 31.26 and 19.90 respectively) (Table 3). A statistical difference belongs to spleen lymphocytes where after 12h of incubation, DG isolated lymphocytes have significant higher PMA-stimulated oxidative activity compared to their counterparts isolated by HL (Stimulation index 6.80 and 2.88 respectively, *p= 0.0354*) (Table 3). Isolated head–kidney leukocytes highlighted the highest PMA-induced oxidative activity. This observation concerns essentially HL-isolated cells but no significant statistical difference was observed between isolation methods (Table 3). After 12 h of incubation, stimulation index indicating the PMA-induced oxidative activity in leukocytes were low for all cell types. In a general way, PMA-induced oxidative activity measured in blood leukocytes was in a same order of magnitude for the two cell isolation mode. The only difference was observed for DG-isolated lymphocytes which had higher PMA-induced oxidative activity after 12h incubation in comparison with HL-isolated lymphocytes (Stimulation index: 2.90 and 1.40 respectively, *p=0.0464*) (Table 3).

3.8. *Phagocytosis:*

It is important to mention here that the analysis of phagocytosis by flow cytometry doesn't allow to discriminate this cell activity at the cell subtype scale. The latex microsphere

ingestions are responsible for modification of cell morphometric parameters (size and granularity).

Head-kidney leukocytes purified with DG had higher phagocytic activities than the same cells isolated by HL (66.35% and 42.44% respectively, *p=0.0432*). This observation was only valid when cell activities were taken as a result of cell isolation with no incubation (Table 4).

Only leukocytes of head-kidney show less score with HL (7.41 compared to DG method (8.76) with statistical significant difference (*p=0.0089*) (Table 5).

The mean numbers of ingested beads per phagocytic cells were not modulated neither by the cell isolation protocol nor by the post-isolation incubation time (Table 5). One can simply signal a tendency to a slightly higher number of beads in cells after 12h incubation following their isolation compared to values observed when phagocytosis was observed immediately after cell isolation (no incubation) (Table 5).

3.9. Purification methods, leukocytes distribution and immune responses:

For DG-isolated leukocytes from spleen, granulocytes percentages were positively correlated with PMA-induced oxidative activity in granulocytes immediately after cell isolation (0.663) (Table 6). For HL-isolated splenocytes, significant positive correlation were observed between granulocytes levels and PMA-induced oxidative activity for the three leukocytes subtypes just after their isolation from spleen (Table 6). If the cell were not incubated at 4°C after their isolation by HL, granulocytes percentages were significantly and positively correlated with phagocytic activities of leukocytes in samples (phagocytosis activity 0.697, number of ingested beads 0.806) (Table 6). After 12h of incubation of spleen-isolated leukocytes by DG, positive correlations were observed between lymphocytes percentages and their PMA-stimulated oxidative activity (0.685). After the same treatment, monocytes/macrophages levels in spleen were positively correlated with basal oxidative activities of all leukocytes subtypes (Table 6). After 12h of incubation of spleen-isolated cells by HL, levels in monocytes/macrophages and also in granulocytes were positively correlated

with the two parameters linked to phagocytosis (phagocytic activity and numbers of ingested beads) (0.806 and 0.867 respectively) (Table 6). Such correlations were not observed in leukocytes suspensions obtained after cell isolation with DG (*p=0.0118 and 0.0068* respectively for the two phagocytic immune endpoints) (Table 6).

In head-kidney, lymphocytes levels obtained by the use HL method show positive correlation with all the basal oxidative activities measured separately in each leukocytes subtypes after 12h of cell incubation at 4°C (lymphocytes: 0.782, monocytes/macrophages: 0.818, granulocytes: 0.806) (Table 6). This result was not observed for DG-isolated leucocytes with significant differences between the two protocols used. (*p=0.044, 0.0174 and 0.0320* respectively) (Table 6). In blood tissue, lymphocytes percentages have positive correlations with PMA-induced oxidative activity in the three leukocytes subtypes after 12h of incubation of cell suspension at 4°C using DG method (lymphocytes: 0.767, monocytes/macrophages: 0.900, granulocytes: 0.817) (Table 6). Such observation was not found in blood-isolated cells with HL protocol used.

4. Discussion:

The objective of our study was to investigate the effects of two cell isolation techniques on cellular responses measured to assess the immune endpoints related to the evaluation of immune status of fish, in different experimental contexts (*ex vivo, in vivo, in situ*). Our observations were realized from three biological compartments classically studied in fish immunology: spleen, head kidney and blood. The thymus, which is important in the immune response of fish, has not been taken into account in our study because of our lack of knowledge of its anatomical location in roach and also of relative low quantities of leucocytes that can be isolated from this organ in two-year-old fish such as those used in our study [34].

One of the difficulties of these analyses lies in the necessity of carrying out measurements on suspensions of live leucocytes extracted from the lymphoid tissues. Teleost fish have the particularity to possess nucleated erythrocytes. These cells are naturally present in the various

tissues of the body. The erythrocytes size, their ovoid shape, the presence of a nucleus and their very large quantities in tissues, disrupt the analyses of leukocyte functionality whatever the techniques used (microscopy, flow cytometry, etc.). It is therefore essential to eliminate erythrocytes from leucocyte suspensions in order to acquire leukocyte response data as close as possible to the functional realities of these cells within the studied organism.

Two leukocyte isolation methods based on the elimination of erythrocytes were studied here, one classically corresponding to a density gradient obtained by centrifugation and a second to date less used and corresponding to a hypotonic lysis of red blood cells [23]. Initially, these methods essentially target the blood compartment. Blood is indeed the obvious tissue to be sampled for leukocyte collection and for which the inconvenience of erythrocyte contamination in leukocyte suspensions is the most important. But interests about the immune responses in spleen and anterior kidney led us to submit them to these same two protocols in order to extract their leukocyte cells.

Obtaining leucocyte suspensions requires several crucial and unavoidable phases [35]. First of all, the tissue must be taken from the whole organism. For the blood, its sampling requires the use of a syringe containing an anticoagulant and allowing for the fish of sufficient size (> 10 cm), the non-invasive blood sampling notably in the caudal vein of the fish [36,37]. For head-kidney and spleen, samples are taken by the organ dissection corresponding to its extraction from the abdominal cavity of the fish. The organ is then dilacerated, mechanically crush in culture medium through a nylon mesh filter (100µm) [33,36]. Blood samples or crushed organs are then processed to remove tissue debris and non-leukocyte cells. In case of separations using centrifugation gradients, the samples are deposited as in our study, on a Ficoll® solution (commercial name of a liquid polyfluorocarbon solution with 1.077 g/mL density) and centrifuged for several minutes. Used speeds and centrifugation times lead to a differential separation of leukocytic cells according to their densities. The red blood cells and debris are found at the bottom of the centrifuge tube and leukocytes are concentrated in a ring shape on Ficoll® surface. After leukocytes recovery, it is necessary to wash them in an excess

of culture medium. This step allows to eliminate residual Ficoll® which may be potentially toxic to the cells [38]. A new centrifugation leading to a cell sedimentation in tube bottom allows to eliminate supernatant containing Ficoll®-contaminated culture medium.

Regarding leukocyte separations based on the removal of erythrocytes by osmotic lysis, the best known methods are used in mammals to remove red blood cells by hyperosmotic shock using ammonium chloride. Such lysis is not valid in teleost due to the nuclei presence in fish erythrocytes which prevent lysis [35]. In 2001, Crippen and colleagues described a technique for removing erythrocytes in trout blood and head-kidney samples by changes in their surrounding environment isotonicity. It is this protocol that we used here with some adaptations for the roach species. Thus, blood samples or crushed organs obtained from the sampling phase were rapidly and transiently exposed to hypotonic conditions (addition of sterile distilled water for 40 seconds). After a rapid isotonicity restoration of medium, cells were centrifuged in order to remove debris allowing cell pellets to be taken up in culture medium. Note that some leukocyte isolation protocols associate osmotic lysis with centrifugation gradient [39]. In our case, these two methods were used separately.

These various steps may constitute physical and chemical stresses for the cells. The question is what are the possible consequences of such stress on the functional analyzes carried out on these cells? In order to limit it, it seems advisable not to do the analyzes immediately after the leukocyte isolation and to subject cell suspensions to a "rest" phase in order to allow them to "recover" from possible traumas created by all the successive phases of the protocols (sampling, crushing, centrifugation, etc.). Therefore, in our study, for the purposes of comparisons, the analyzes were carried out either directly on the freshly isolated leukocytes or on cells incubated for 12 h at low temperature (+4°C) after the isolating procedure.

In fish, if leukocyte distribution data may vary from one species to another and inside the same species, this endpoint is also modulated by the development stage of the organism but also naturally according to its parasitic status [9,40].

In cyprinids and particularly in common carp (*Cyprinus carpio*), leukocyte densities are as follow: lymphocytes (1.020 g/mL), monocytes / macrophages (1.070 g/mL) and granulocytes (1.083 g/mL) [41]. The Ficoll® density being 1.077 g/mL, it is then understandable that leukocyte isolation, based on the use of a Ficoll® gradient, leads to the isolation of lymphocytes and monocytes / macrophages cell types [42]. That's confirmed by our results. Compared to the density gradient, hypotonic lysis is associated with leukocyte enrichments, which essentially concern granulocytes for the three tissues targeted in our study. This result is particularly noticeable for leukocyte suspensions isolated from head kidney where granulocytes are naturally abundant [16]. However, the head kidney is also a tissue where there is a production of lymphocytes, particularly B lymphocytes [43,44]. More recently, the use of marked antibodies specific for certain cell types, has shown that density gradients alone can influence the leukocyte composition of blood compared to data acquired on whole blood [45].

The higher collection of lymphocytes obtained with DG method may trigger shifts in cells reaction as our results show correlation between lymphocytes amounts and oxidative activity in the whole cells suspensions in blood and head-kidney noticeable after a period of cells incubation (Table 6). In spleen, this phenomenon is observable with monocyte/macrophages cells. As a matter of fact, Ulmer *et al*. (1984) showed a density modulation of mammalian monocytes using Ficoll® separation that grants them same density as lymphocytes, a difficulty in defining leucocytes sub-populations when using flow cytometry. After 12h of post-isolation incubation, differences disappear which confirm our advice on the use of cell incubation before their cytometric analysis.

The granulocyte enrichment of leukocytes suspensions after HL isolation method was confirmed by positive correlations on granulocytes amounts in head-kidney samples and the related high phagocytic activity of leukocytes. This increase in phagocytosis in cell suspensions obtained after leukocyte isolation by HL could be explained by increase of cell debris in cell suspensions by the use of this technique. Therefore, Zhou *et al.* (2012) stress out

that cell ghosts and debris are subject to phagocytosis by phagocytic cells after a lysis purification method.

Data of leukocytes distributions in lymphoid tissues depend very much on the leukocyte isolation modalities (Table 7).

Our leukocyte distribution data and those obtained in literature are clearly modulated by the choice of the leukocyte isolation protocol (especially for spleen and head-kidney leukocytes) (Table 7).

However, such influence was not observed for isolated trout leukocytes using protocols that were relatively similar to those used in our study [23] (Table 7). Whether acquired by Percoll® discontinuous density gradients or by histopaque-1077 density gradients or by the use of hypotonic lysis, leukocyte compositions are found to be similar in rainbow trout (*Oncorhynchus mykiss*) whatever the isolating protocol used [23] (Table 7). Such a result is surprising insofar as the two types of gradient used by these authors and similar to ours, allow to isolate the mononucleated cells, namely lymphocytes and monocytes / macrophages.

An important variability is observed in leucocyte distributions between the two incubation conditions (no incubation or 12 h of incubation at 4 ° C.) to which the cells are subjected after their isolation by hypotonic lysis. This variability in cell types' proportions was observed for the spleen and head kidney leukocyte suspensions. It could be due to the cell heterogeneity in these two organs and to the various sensitivities of cells which constitute them to the osmotic stress undergone during the isolation phase. Indeed, studies show that leukocyte sensitivity to osmotic stress results in oxidative stress altering their long-term survival [60]. It should be noted that in our study, hypotonic lysis as well as density gradient, has no significant effect on leucocyte mortality, regardless of the tissue from which these leukocytes are derived. However, it can be noted that method to estimate cell mortality we used (labeling cell with propidium iodide) only takes into account the cell deaths by necrosis and not those due to apoptosis. Thus, the variability observed over time in leucocyte distributions of cell isolated

after hypotonic lysis of erythrocytes and incubated for 12h at 4 ° C could be linked to destabilizations in certain leukocyte categories such as lymphocytes. However, as head-kidney is considered as a leukopoietic organ with more granulocytes content and naïve cells than spleen or blood, a cell proliferation may be possible explaining difference observed during incubation time. We can noticed that the use of Ficoll® gradient was associated with a better stability in cellular distributions observed between the two incubation times.

The studies using continuous density gradients (with Ficoll® or Percoll®: densities 1.077 and 1.075 g / mL respectively) are relatively numerous [9,10,22,61]. The relative small amounts of granulocytes recovered by this type of leukocyte isolation may limit observations on lymphocytes-related functions, such as the adaptive response of antibody production [62,63]. Thus, the use of hypotonic lysis must lead to a better vision of the overall fish immune response, taking into account, besides adaptive immunity, of natural immunity with a best isolation of granulocyte cells [64,65]. In addition, taking into account the different leucocyte subpopulations is very important as the tissue leukocyte distribution (particularly blood) constitutes a strong marker of stressed organism in vertebrates [66]. The lymphocyte / granulocyte ratio favorable to lymphocytes in the normal body is modified in favor of granulocytes when the fish are stressed [57,67].

Since the two protocols used in our study led to differences in leucocyte distributions for the three studied tissues, implications in responses of studied immune functions were expected.

The effects of the two modes of cell isolation were observed on two major functions of the native fish immunity, phagocytosis and basal/stimulated leukocyte oxidative activities. The effects on cell mortality were analyzed as this data is important for interpretation of cellular functional responses. The two treatments did not induce significant mortality, the most notable effects appearing for the cells which have just been isolated. Therefore, the cell incubation for 12 hours at 4 ° C may reduce the potential deleterious effects of the isolating procedures.

The isolation of the leukocytes by density gradient leads to a transient increase in basal oxidative activity which disappears after 12h of cell incubation at +4°C. This increase essentially concerns splenocytes. The blood leukocytes isolated by this same method exhibit high basal oxidative activities after 12 h of incubation at 4 ° C and for the three leukocyte types (lymphocytes, monocytes and granulocytes). When leukocytes are exposed to PMA for stimulation of intracellular ROS production, head kidney cells isolated by hypotonic lysis show the highest stimulation levels. This stimulation disappears after 12h of cell incubation at +4°C. A same phenomenon is observed for blood leukocytes isolated by hypotonic lysis but only immediately after the isolation phase.

Thus, such observations reveal that density gradient isolation leads to a transitory increase in basal ROS production in spleen lymphocytes whereas hypotonic lysis is associated with an increase in PMA-induced ROS production especially in head kidney leukocytes.

These increases in cell oxidative activities, particularly those induced by PMA, are consistent with increases in granulocyte proportions observed in leukocyte suspensions obtained by hypotonic lysis. Expectedly, granulocytes are the cells that exhibit the highest oxidative activities in comparison with other cell types. This observation is consistent with the functional capacities of these cells, which play an important role in inflammatory process [68,69]. The stronger stimulation in PMA-induced oxidative activity observed in head kidney leukocytes was not due to a cell priming induced by the isolation protocol but to the increase in granulocytes amounts in the Leucocyte suspensions obtained.

If effects are observed on cellular oxidative activities, no effect appears on phagocytosis. A low tendency of decrease is observed for leukocytes isolated by hypotonic lysis. Such a result is surprising given the increased presence of granulocytes in leukocyte suspensions obtained by hypotonic lysis. Although macrophages are important phagocytic cells, granulocytes have an intense phagocytosis activity in numerous fish species [70,71]. The persistence in phagocytosis activity of cells isolated by erythrocytes hypotonic lysis and its non-stimulation

related to increase in granulocyte percentages, could be explained by a partial inhibition of this cell function by conditions linked to the leukocyte isolation procedure used. In our study, the hypotonic lysis protocol used consists in a rapid cell exposure to cold distilled water. Cell exposition to such conditions could explain the inhibition in cellular phagocytosis activity. Another explanation could be related to the fish species studied. In roach, phagocytosis could be carried out by myeloid cells (monocytes/macrophages and granulocytes) but also by B lymphocytes [72,73]. Since the two isolation protocols used have a limited influence on the lymphocyte levels in cell suspensions, this leads to a relative stability in phagocytosis activity whatever the isolation treatment of cells.

Whatever the isolation method used, our results show their effects on intracellular ROS production. These oxidative activations sometimes only concern some leukocyte types (spleen lymphocytes and monocytes/macrophages) or in other cases all populations (particularly blood leukocytes) are concerned. For this last point, hypotonic lysis leads to a transient increase in PMA-stimulated oxidative activity of head kidney leukocytes whereas isolation by density gradient increases the oxidative activity of blood leucocytes. It is important to note that our results had some limitations concerning for instance the relative few numbers of observations (N=10) which may depend also on the physiological status of fish used (2 years old fish, in reproduction phase of their biological cycle and with no apparent parasitism).

Within limits of our experimental conditions, the spleen is the organ whose leucocyte oxidative activities (stimulated or not) are only slightly influenced by the methods used for leukocyte isolation. This is also the case for the anterior kidney, but for this tissue, it is necessary to incubate the isolated cells for 12 h at +4°C.

Thus, our study improves knowledge about the effects of two protocols used for leukocyte isolation on cell functions very commonly analyzed in fish immunology. In the balance sheet, each of the two methodologies used has advantages and disadvantages. The hypotonic lysis allows to isolate a greater variety of leukocytes types whereas the density gradient used

ensures a better stability of cells distributions over time. Finally, whatever the method used, a recovery of cells following their isolation (for instance 12 h at +4°C. as in our study) is required in order to improve quality of obtained results.

To date, studies in which the hypotonic lysis protocol has been used are few. Data on its effects on cell functions are still limited and further works should be carried out to better understand the impact of this type of leukocyte isolation procedure depending on targeted fish species.

It is important to note out that results in leukocyte separation in terms of quantities and qualities of isolated cell types are influenced by all the different steps required for cell isolation. For the same fish species and for the same tissue, Ficoll® use may be associated with different centrifugation times and rates leading to differences in leukocytes distributions obtained [35] (Table 7). Thus, it is the set of two protocols used which may influence results and which must be taken into consideration during acquired data analysis. Other protocols for leukocyte isolation exist and our study does not pretend to be complete on this subject. Among the selection criteria for leukocyte isolation protocols, logistical constraints associated with experiments must also be taken into account. Sometimes, this is the case in studies carried out in eco-immunology and eco-physiology on fish species of wild fauna. Some trade-offs must be done between technical feasibility of experiments which should not be too long in order to ensure the optimum conservation of living cell samples and the statistical requirements in terms of quantity of observations required to ensure validity of results.

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Figure captions

2 columns fitting image

Figure 1. Gating method for cytograms analysis. Example for head-kidney leukocytes isolated by density gradient or hypotonic lysis. Head-kidney leukocytes were purified by density gradient (A, B, C) or hypotonic lysis (D, E, F). P1 = Leukocytes gate (A and D); P2 = Gate of excluded doublets in FSC-H/FSC-A cytograms (B and E); P3 = impurities (C and F); Leukocytes were recorded in P1 gate with P2 and P3 gates exclusion from counts (Total of 10000 events in P1).

Single column fitting image

Figure 2. Gating method used to determine leukocyte subpopulations distribution in cell suspensions. Leukocytes subpopulations were gated as lymphocytes + thrombocytes (Ly), monocytes/macrophages (M) and granulocytes (Gr).

Single column fitting image

Figure 3. Cell mortality measurement. A - The pick in the left part was related to viable leukocytes autofluorescence. Dead or senescent cells were positively marked with Propidium

iodide. Cell mortality percentages were calculated and corresponded to fluorescence of PI marked cells. B - PI-marked cells were colored in red within the total leukocytes population.

1.5 column fitting image

Figure 4. Phagocytosis assay. A - Fluorescence of cells ingesting one bead (mean fluorescence in M1) or 3 beads and more (mean fluorescence in M2). B - dotplots presentation of cells ingesting one bead (blue) and cells ingesting 3 beads and more (red). Uncolored ones represent cells ingesting two beads.

1.5 column fitting image

Figure 5. Oxidative activity assay. A, B - *Mean fluorescence of DCFH measured in head-kidney lymphocytes (blue), in monocytes/macrophages (pink) and in granulocytes (green). C, D - Mean fluorescence of DCFH measured in cells unstimulated or stimulated with PMA in function of their complexity (SSC-A).*

Whole page fitting image

Figure 6. Leukocytes cytograms of spleen, head-kidney and blood tissues in function of purification methods. Leukocytes from spleen, head-kidney and blood cell suspensions were purified by density gradient or by hypotonic lysis of erythrocytes. After cell isolation, samples were diluted to 1:10 in PBS and analysed for leukocytes composition by flow cytometry using FSC (size) / SSC (complexity) parameters. Cytograms were acquired just after cells purification (no incubation) and after 12h of cell incubation.

2 column fitting image

Figure 7. Sub-populations leukocytes distributions in spleen, head-kidney and blood tissues in function of cell purification methods. Stacked bars represent leukocytes composition of tissue either purified by density gradient (DG) or by hypotonic lysis of erythrocytes (HL). Cell suspension analysis were realized immediately after isolation procedures (0h) or after a 12h of incubation of cell suspension before analysis (12h). Bar height represent median of n=10 observations and the total bar height represent 100% of each leukocytes population.

Dark-grey bars correspond to lymphocytes, grey to monocytes/macrophages and dark ones to granulocytes. Asterisks above bars indicate significant differences between data obtained for the two purification methods ($p<0.05$)

2 columns fitting image

Figure 8. Leukocytes mortality in spleen, head-kidney and blood tissues in function of cell purification methods. Cell necrosis was measured by flow cytometry as percentages of PI-positive cells for total leukocytes population (T) or for each subpopulations: lymphocytes (L), monocytes/macrophages (M) and granulocytes (G) isolated from spleen (A), head-kidney (B) and blood (C). For each tissue, cell suspensions were separated in two equal volumes and purified either by density gradient (dark grey boxes) or by hypotonic lysis of erythrocytes (grey boxes). Boxplots represent 25th and 75th percentiles over and below the median (line within the box) for n=10 observations. Bars at the top and bottom of boxes indicate 10th and 90th percentiles. Cell suspension analysis were realized immediately after isolation procedures (0h) or after a 12h of incubation of cell suspension before analysis (12h). Asterisks above bars indicate significant differences between data obtained for the two purification methods (*: $p<0.05$; **: $p<0.01$).

2 column fitting image

Figure 9. Basal intracellular ROS in spleen, head-kidney and blood leukocytes in function of cell purification methods. Basal intracellular ROS were measured by flow cytometry as mean fluorescence of DCF in total leukocytes population (T) or for each leukocyte subpopulations: lymphocytes (L), monocytes/macrophages (M) and granulocytes (G) isolated from spleen (A), head-kidney (B) and blood (C). For each tissue, cell suspensions were separated in two equal volumes and purified either by density gradient (dark grey boxes) or by hypotonic lysis of erythrocytes (grey boxes). Boxplots represent 25th and 75th percentiles over and below the median (line within the box) for n=10 observations. Bars at the top and bottom of boxes indicate 10th and 90th percentiles. Cell suspension analysis were realized immediately after isolation procedures (0h) or after a 12h of incubation of cell suspension

before analysis (12h). Asterisks above bars indicate significant differences between data obtained for the two purification methods (*: $p<0.05$; ***: $p<0.001$).

Tables:

Table 1. Total cell numbers (cell per mL) obtained after leukocytes isolation in spleen, head-kidney and blood tissues. Cell numerations were realized by flow cytometry on total leukocyte cell suspensions isolated from spleen, head-kidney and blood. For each tissue, cell suspensions were separated in two equal volumes and purified either by density gradient or by hypotonic lysis of erythrocytes. Numeric data are median and min and max values obtained from n=10 observations. Cell suspension analysis were realized immediately after isolation procedures (0h).

	Density gradient			**Hypotonic lysis**		
	Median	Min	Max	Median	Min	Max
Spleen	$5.26x10^6$	$1.34x10^6$	$7.85x10^6$	$4.40x10^6$	$0.72x10^6$	$10.0x10^6$
Head-kidney	$8.93x10^6$	$4.69x10^6$	$20.4x10^6$	$14x10^6$	$7.83x10^6$	$24.1x10^6$
Blood	$0.57x10^6$	$0.004x10^3$	$4.55x10^6$	$0.36x10^6$	$0.02x10^6$	$1.24x10^6$

Table 2. Samples purity ~~after isolation~~. Results were expressed as number of cells per millilitre. Numeric data are median and min and max values obtained from n=10 observations. Cell suspension analysis were realized immediately after isolation procedures (0h). Asterisk indicates statistical significant difference ($p<0.05$) between the two purification

961 methods right after purification. Significant differences are indicated with underlined medians
962 on the same line.

	Density gradient			*Hypotonic lysis*		
	Median	Min	Max	Median	Min	Max
Spleen	97.6%*	93.0%	99.0%	94.8%	91.9%	97.5%
Head-kidney	96.7%*	95.2%	97.3%	94.7%	92.7%	96.0%
Blood	97.4%*	90.3%	99.0%	89.2%	74.6%	97.3%

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967 **Table 3.** ***Activated intracellular ROS production in spleen, head-kidney and blood***
968 ***leukocytes in function of cell purification methods.*** Basal and PMA-activated intracellular
969 ROS were measured by flow cytometry in total leukocytes population or for each leukocyte
970 subpopulations (lymphocytes, monocytes/macrophages and granulocytes) isolated from
971 spleen, head-kidney and blood. For each tissue, cell suspensions were separated in two equal
972 volumes and purified either by density gradient or by hypotonic lysis of erythrocytes. The
973 results were expressed in stimulation index as the ratio between the mean fluorescence
974 measured in stimulated cells (DCFH-DA + PMA) and the basal mean fluorescence of control
975 (DCFH-DA only). Numeric data are median and min and max values obtained from n=10
976 observations. Cell suspension analysis were realized immediately after isolation procedures
977 (0h) or after a 12h of incubation of cell suspension before analysis (12h). Asterisks indicate
978 significant differences between data obtained for the two purification methods (*: $p<0.05$).
979 Significant differences are indicated with underlined medians on the same line.
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		Cells	*Density gradient*			*Hypotonic lysis*		
			Median	Min	Max	Median	Min	Max
Spleen	**No incubation**	Total leucocytes	7..37	0.71	14.29	6.52	2.75	25.56
		Lymphocytes	6.25	0.64	11.73	4.25	2.14	13.27
		Monocytes / Macrophages	5.12	0.76	10.41	4.24	2.02	18.86
		Granulocytes	31.26	1.92	86.59	19.90	5.30	61.38
	12h incubation	Total leucocytes	6.73	3.04	13.94	5.60	2.18	7.50
		Lymphocytes	6.80*	3.16	13.33	2.88	1.58	6.55
		Monocytes / Macrophages	5.64	2.31	12.90	2.56	1.64	5.60
		Granulocytes	10.23	1.59	65.15	9.66	3.84	29.67
Head-kidney	**No incubation**	Total leucocytes	23.38	12.04	113.75	48.78	18.01	160.38
		Lymphocytes	20.18	6.83	92.17	33.34	10.96	124.03
		Monocytes / Macrophages	14.32	6.08	78.06	29.45	9.94	110.61
		Granulocytes	41.56	19.01	249.32	60.97	28.95	170.86
	12h incubation	Total leucocytes	4.32	2.47	19.48	7.28	0.88	87.32
		Lymphocytes	5.55	1.81	17.36	6.00	1.07	30.93
		Monocytes / Macrophages	3.11	2.43	14.95	5.08	1.29	40.13
		Granulocytes	4.47	1.46	27.99	8.94	0.50	110.47
Blood	**No incubation**	Total leucocytes	4.66	2.36	24.34	8.24	1.58	28.15
		Lymphocytes	3.59	1.64	23.19	2.38	1.55	5.62
		Monocytes / Macrophages	6.99	1.26	24.03	4.05	1.79	13.38
		Granulocytes	9.18	2.43	208.39	21.67	7.97	151.05
	12h	Total leucocytes	2.58	0.87	7.49	4.53	1.89	11.38

incubation	Lymphocytes	2.90*	0.88	7.17	1.40	1.02	2.39
	Monocytes / Macrophages	1.45	0.88	5.57	1.77	0.93	2.93
	Granulocytes	11.16	0.17	19.36	12.03	3.81	43.56

Table 4. ***Phagocytosis activity of spleen, head-kidney and blood leukocytes in function of cell purification methods.*** The phagocytic activity corresponds to percentages of cell ingesting three beads and more. Phagocytic activity was measured by flow cytometry for total leukocytes population~~population or for each leukocyte subpopulations (lymphocytes, monocytes/macrophages and granulocytes)~~ isolated from spleen, head-kidney and blood. For each tissue, cell suspensions were separated in two equal volumes and purified either by density gradient or by hypotonic lysis of erythrocytes. Numeric data are median and min and max values obtained from n=10 observations. Cell suspension analysis were realized immediately after isolation procedures (0h) or after a 12h of incubation of cell suspension before analysis (12h). Asterisks indicate significant differences between data obtained for the two purification methods (*: $p<0.05$). Significant differences are indicated with underlined medians on the same line.

		Density gradient			***Hypotonic lysis***		
		Median	Min	Max	Median	Min	Max
Spleen	No incubation	61.58%	23,64%	84,78%	55,32%	18,91%	82,92%
	12h incubation	67,09%	49,77%	79,49%	73,65%	56,35%	80,51%
Head-kidney	No incubation	66,35%*****	29,17%	87,96%	42,44%	27,46%	64,10%

	12h incubation	68,65%	48,89%	83,32%	57,75%	36,01%	78,55%
Blood	No incubation	65,86%	37,46%	97,37%	46,70%	34,16%	77,42%
	12h incubation	60,92%	51,61%	65,72%	53,63%	44,82%	92,95%

Table 5. ***Numbers of ingested beads in spleen, head-kidney and blood leukocytes in function of cell purification methods.*** The number of phagocytosed beads was calculated by dividing the mean fluorescence of events corresponding to three and more beads-ingesting cells by the fluorescence of events corresponding to only one bead-ingesting cells. Numbers of ingested beads was measured by flow cytometry for total leukocytes population ~~or for each leukocyte subpopulations (lymphocytes, monocytes/macrophages and granulocytes)~~ isolated from spleen, head-kidney and blood. For each tissue, cell suspensions were separated in two equal volumes and purified either by density gradient or by hypotonic lysis of erythrocytes. Numeric data are median and min and max values obtained from n=10 observations. Cell suspension analysis were realized immediately after isolation procedures (0h) or after a 12h of incubation of cell suspension before analysis (12h). Asterisks indicate significant differences between data obtained for the two purification methods (*: $p<0.05$). Significant differences are indicated with underlined medians on the same line.

		Density gradient			**Hypotonic lysis**		
		Median	Min	Max	Median	Min	Max
Spleen	No incubation	6.67	4.94	9.45	6.80	4.86	8.51

	12h incubation	8.44	7.79	9.11	8.89	7.12	9.85
Head-kidney	No incubation	7.43	5.18	8.93	6.56	4.91	7.68
	12h incubation	8.76**	5.44	10.43	7.41	5.72	8.52
Blood	No incubation	7.08	6.00	11.15	7.27	6.46	9.63
	12h incubation	7.99	6.83	8.86	8.70	5.10	12.06

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Table 6. Correlations (ρ of Spearman) between leukocytes distributions and cell immune responses in function of cell purification methods. Values of correlation coefficients were significant at $p<0.05$ (bold characters). After Fisher transformation, differences between ρ values were evaluated with a Z-test and were noticed as follow (* $p<0.05$ no incubation vs 12 incubation for same tissues and purification methods; # $p<0.05$ density gradient vs hypotonic lysis for same tissues and incubation time). Ly.=Lymphocytes; M.=Monocytes/macrophages; Gr.=Granulocytes; Phago=Phagocytosis.

		Basal oxidative activity (Ly.)	Basal oxidative activity (M.)	Basal oxidative activity (Gr.)	Stimulated oxidative activity (Ly.)	Stimulated oxidative activity (M.)	Stimulated oxidative activity (Gr.)	Phago %	Numbers of ingested beads
SPLEEN - Density gradient									
No incubation	Ly.%	-0.091	0.115	0.370	-0.152	-0.164	-0.358	-0.212	-0.236
	M.%	-0.079	-0.285	-0.285	0.079	0.018	0.164	-0.248	-0.176
	Gr.%	0.517	0.274	-0.128	0.529	0.529	**0.663**	0.517	0.492
12h incubation	Ly.%	**-0.818**	**-0.745**	**-0.745** *	**0.685**	**0.709**	**0.648** *	**0.685**	0.382
	M.%	**0.818** *	**0.745** *	**0.745** *	**-0.685**	**-0.709**	**-0.648**	**-0.685**	-0.382
	Gr.%	0.128	0.061	0.116	-0.043	-0.116	0.055	0.097	-0.328
SPLEEN - Hypotonic lysis									
No incubation	Ly.%	0.382	0.273	0.394	**-0.661**	**-0.648**	**-0.636**	**-0.636**	**-0.733**
	M.%	**0.770**	0.455	0.479	-0.600	-0.588	-0.612	-0.600	-0.442
	Gr.%	-0.430	-0.164	-0.285	**0.770**	**0.758**	**0.733**	**0.697**	**0.806**
12h incubation	Ly.%	-0.055	0.018	-0.103	**0.745** *	0.539 *	-0.333	0.273	**-0.830** #
	M.%	0.188	0.079	0.285	-0.576	-0.455	0.261	-0.321	**0.806** *#
	Gr.%	-0.018	-0.091	-0.067	**-0.830** * #	**-0.636** *	0.382	-0.127	**0.867** #
HEAD KIDNEY - Density Gradient									
No incubation	Ly.%	-0.103	-0.139	-0.115	0.248	0.248	0.285	0.188	0.261
	M.%	-0.297	-0.273	-0.309	0.200	0.103	0.139	-0.224	-0.261
	Gr.%	-0.006	0.018	0.006	-0.115	-0.115	-0.139	-0.164	-0.200
12h incubation	**Ly.%**	-0.115	-0.261	-0.139	-0.333	-0.176	0.079	0.212	0.236
	M.%	-0.539	-0.576	-0.479	-0.539	-0.600	-0.624	-0.345	-0.273
	Gr.%	0.188	0.285	0.248	0.430	0.345	0.139	0.091	-0.067
HEAD KIDNEY - Hypotonic lysis									
No incubation	Ly.%	-0.018	0.345	0.564	-0.176	-0.297	0.139	0.515	0.430
	M.%	-0.115	-0.564	**-0.709**	-0.030	-0.079	-0.285	-0.624	-0.552
	Gr.%	0.030	-0.321	-0.600	0.079	0.309	-0.176	-0.515	-0.455
12h incubation	Ly.%	**0.782** #	**0.818** #	**0.806** #	-0.382	**-0.733**	**-0.794** * #	**-0.891** *#	0.200
	M.%	**-0.636**	-0.624	**-0.636**	0.152	0.503 #	0.527	0.503 *	**-0.709**
	Gr.%	**-0.588**	**-0.636**	-0.612	0.442	0.612	**0.709**	**0.745** *	0.055

1039 ***Table 6. Continued***

		Basal oxidative activity (Ly.)	Basal oxidative activity (M.)	Basal oxidative activity (Gr.)	Stimulated oxidative activity (Ly.)	Stimulated oxidative activity (M.)	Stimulated oxidative activity (Gr.)	Phago %	Numbers of ingested beads
BLOOD - Density Gradient									
No incubation	Ly.%	-0,442	-0,418	-0,333	-0,539	-0,309	0,103	0,018	-0,139
	M.%	0,503	0,442	0,127	0,588	0,248	0,030	-0,333	-0,236
	Gr.%	-0,224	-0,139	0,285	0,188	0,333	-0,467	0,467	0,552
12h incubation	Ly.%	0,600 *	**0,667** *	0,350	**0,767**	**0,900**	**0,817**	0,567	-0,067
	M.%	-0,600 *	**-0,717** *	-0,200	**-0,800**	**-0,933**	**-0,833**	-0,500	-0,033
	Gr.%	-0,600	-0,600	-0,383	**-0,700**	**-0,750**	-0,650	-0,483	0,033
BLOOD – Hypotonic lysis									
No incubation	Ly.%	-0,217	0,000	0,083	-0,417	-0,183	-0,050	0,188	0,127
	M.%	0,633	-0,133	-0,283	0,017	-0,250	-0,267	-0,455	-0,418
	Gr.%	-0,233	0,417	0,100	0,383	0,650	0,567 #	0,042	0,115
12h incubation	Ly.%	0,000	0,071	-0,310	0,238	-0,286 #	0,071	0,285	0,248
	M.%	0,548 #	-0,143	0,619	-0,667	0,095 #	-0,190	-0,358	-0,261
	Gr.%	-0,238	0,238	0,381	0,000	0,381 #	0,000	-0,079	-0,079

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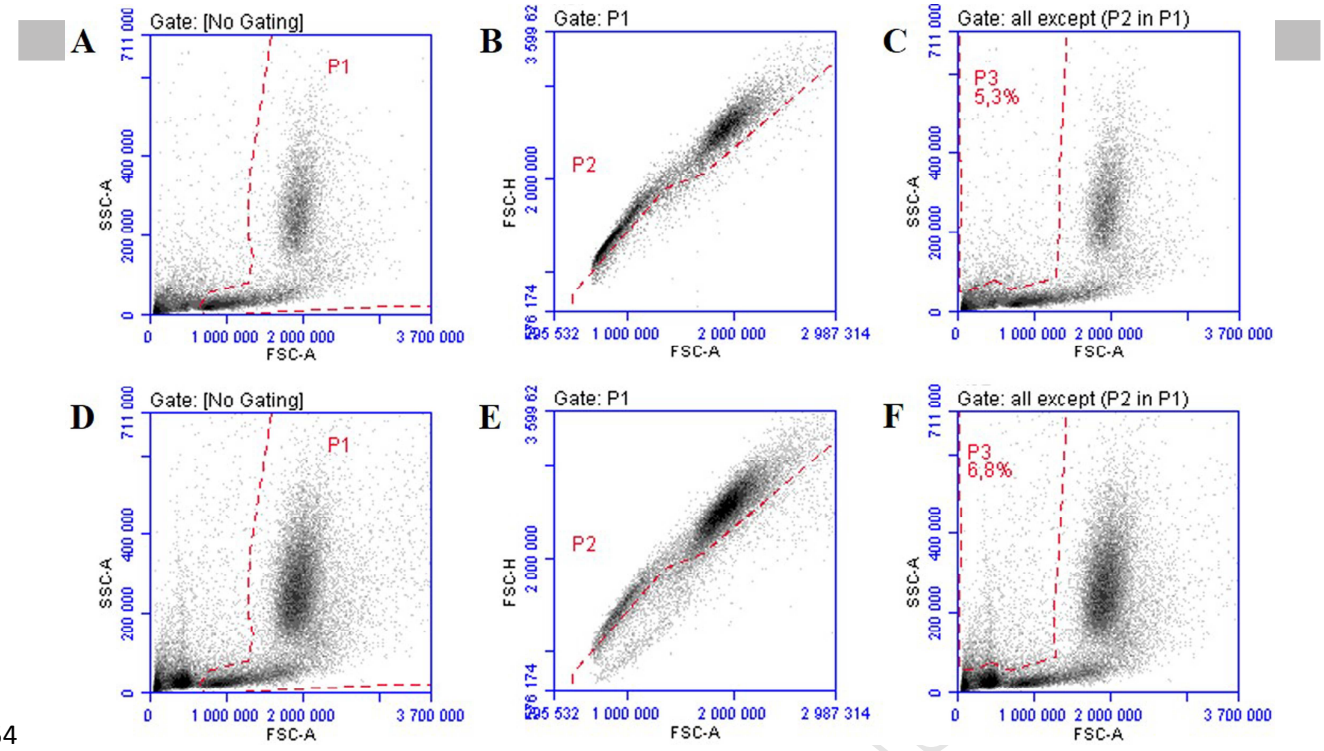
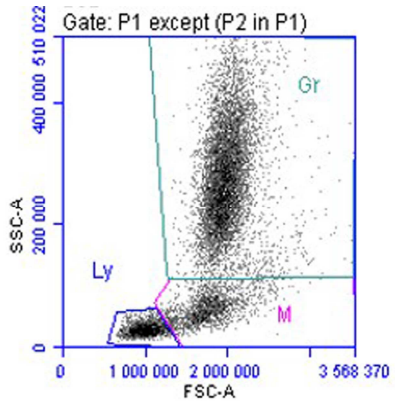
1052 ***Table 7. Partial bibliographic review of leukocyte distribution data in teleost lymphoid organs in function of cell purification methods.*** N.E: non evaluated. Results obtained in the
1053 present study were indicated in bold characters

Tissue	Fish species	Separation type	Lymphocytes %	Monocytes/ macrophages%	Granulocytes%	References
Spleen	*Gasterosteus aculeatus*	Organ crushing (Flow cytometry)	53.91-86.21			Le Guernic *et al.* 2016 [48]
	Cottus sp.	Organ crushing (Flow cytometry)	32.3-85.1	14.9-67.7		Bado-Nilles *et al.* 2015 [49]
	Oncorhynchus mykiss	Sepracell-MN (1.062-1.098)	10-98	0.0-13	1.8-50	Congleton *et al.* 1990 [16]
	Rutilus rutilus	Organ crushing (Flow cytometry)	82-88	12-18		Gagnaire *et al.* 2015 [50]
	Rutilus rutilus	**Ficoll® (1.077)**	**82.34**	**13.14**	**2.24**	**Samai *et al.***
	Rutilus rutilus	**Hypotonic lysis (distilled water)**	**48.64**	**17.75**	**31.04**	**Samai *et al.***
Head-kidney	*Oncorhynchus mykiss*	Sepracell-MN (1.062-1.098)	18-68	0.8-33	18-57	Congleton *et al.* 1990 [16]
	Oncorhynchus mykiss	Hypotonic lysis (distilled water)	79.4	6	14.3	Crippen *et al.* 2001 [23]
	Oncorhynchus mykiss	Percoll® (1.048-1.070)	75.1	5.3	18.8	Crippen *et al.* 2001 [23]
	Oncorhynchus mykiss	Ficoll® (1.077)	N.E.	54.7		Chilmonczyk and Monge 1999 [9]
	Cyprinus carpio	Percoll® (1.020-1.093)	62	18	20	Kemenade *et al.* 1994 [41]
	Rutilus rutilus	**Ficoll® (1.077)**	**65.29**	**12.33**	**19.58**	**Samai *et al.***
	Rutilus rutilus	**Hypotonic lysis (distilled water)**	**40.09**	**15.98**	**40.80**	**Samai *et al.***
Blood	*Oncorhynchus mykiss*	Histopaque (1.077)	93.1	0.5	6	Crippen *et al.* 2001 [23]
	Oncorhynchus mykiss	Hypotonic lysis (distilled water)	91.9	0.4	7.4	Crippen *et al.* 2001 [23]
	Oncorhynchus mykiss	Total blood (Flow cytometry)	90.61		4.78	Douxfils *et al.* 2017 [51]
	Cyprinus carpio	Smears	96.5		2.5	Witeska *et al.* 2017 [52]
	Oncorhynchus mykiss	Total blood (Flow cytometry)	36.5	5		Korytář *et al.* 2013 [45]
	Oncorhynchus mykiss	Percoll® (1.075)	58.6	5		Korytář *et al.* 2013 [45]
	Ictalurus punctatus	Smears	43	1.6	3.5	Ellsaesser and Clem 1986 [53]
	Oreochromis niloticus	Smears	40.6-41.5	9.3-10.6	47.8-48.9	Perera and Pathiratne 2012 [54]
	Limanda limanda		70	10		Pulsford *et al.* 1994 [55]
	Sebastes oculatus	Smears	95	1.5	3	Sueiro and Palacios 2016 [56]
	Odontesthes bonariensis	Smears	75.3	13.3	9.6	Zebral *et al.* 2015 [57]
	Mugil cephalus	Smears	90	2.5	6.7	Faggio *et al.* 2014 [58]
	Rhamdia quelen	Neubauer chamber			8	Montanha *et al.* 2014 [59]
	Rutilus rutilus	**Ficoll® (1.077)**	**87.66**	**9.74**	**1.45**	**Samai *et al.***
	Rutilus rutilus	**Hypotonic lysis (distilled water)**	**72.85**	**14.22**	**10.74**	**Samai *et al.***



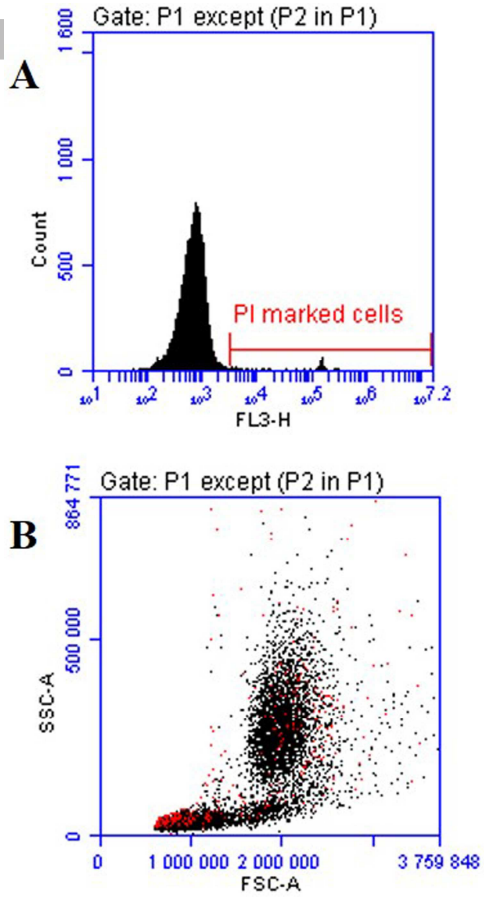
1054

1055 **Fig.1**



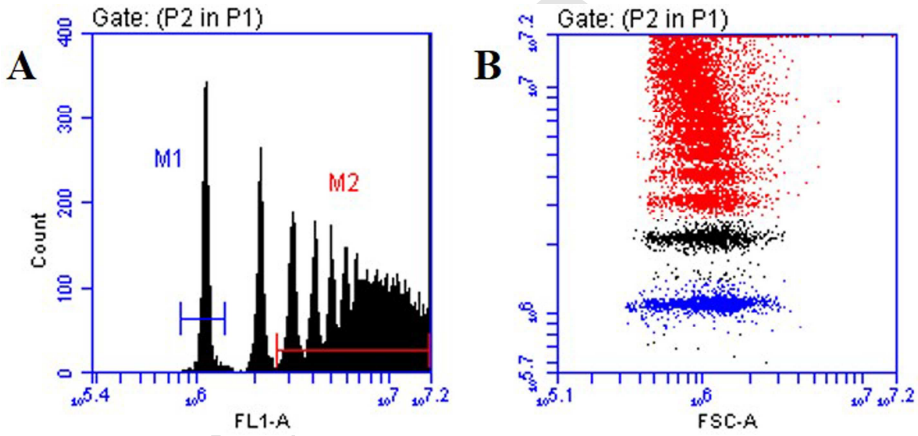
1056

1057 **Fig.2**



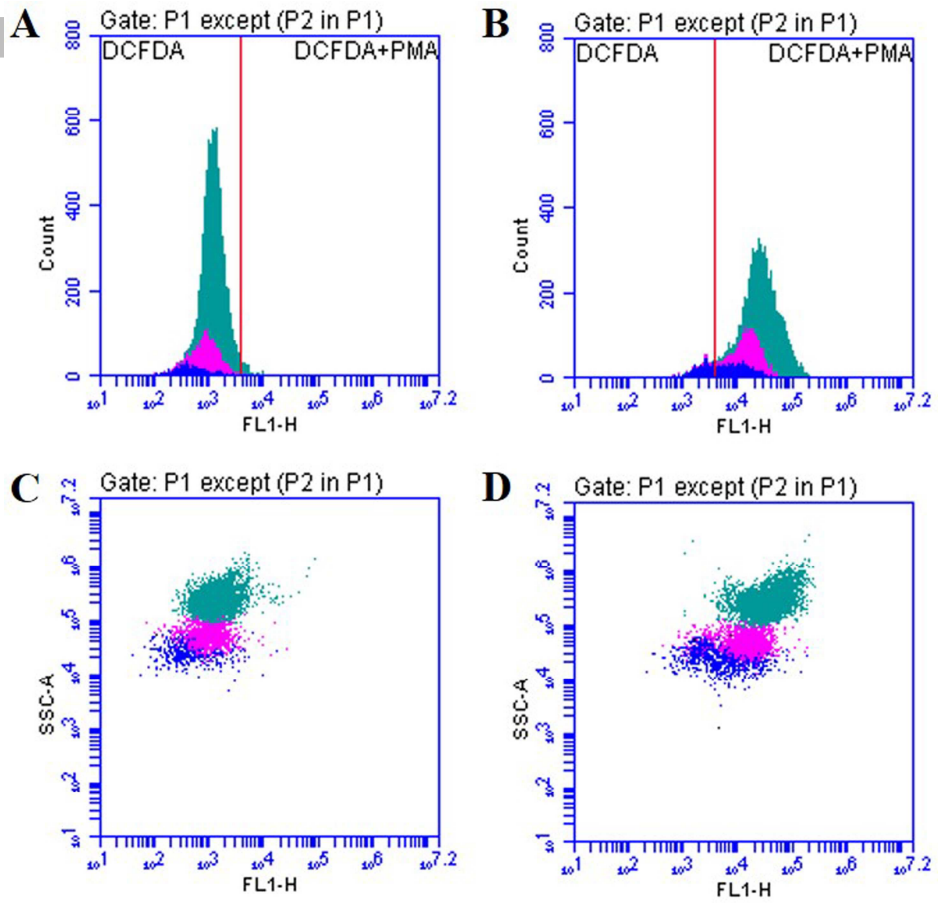
1058

1059 **Fig.3**



1060

1061 **Fig.4**



1062

1063 **Fig.5**

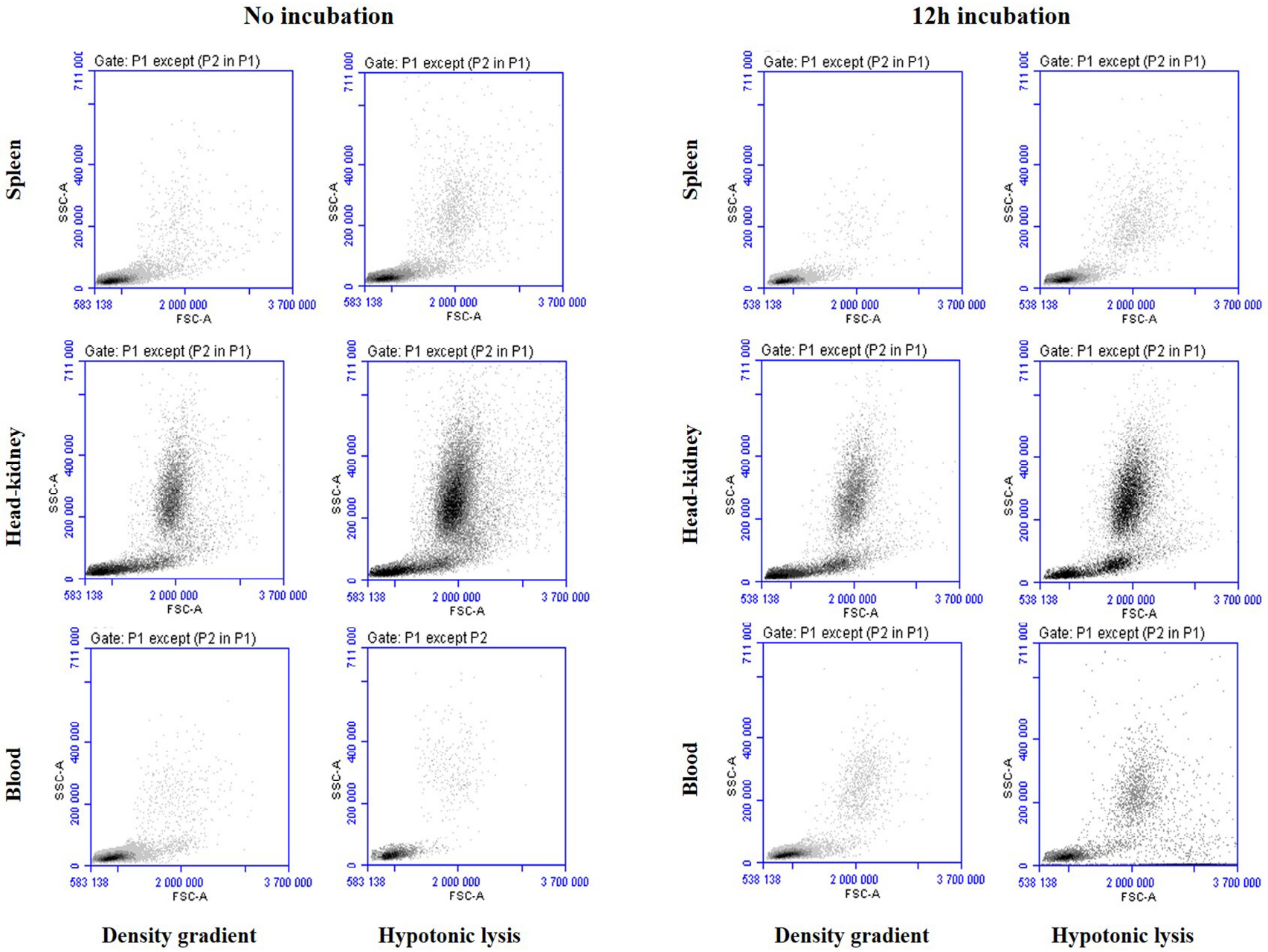
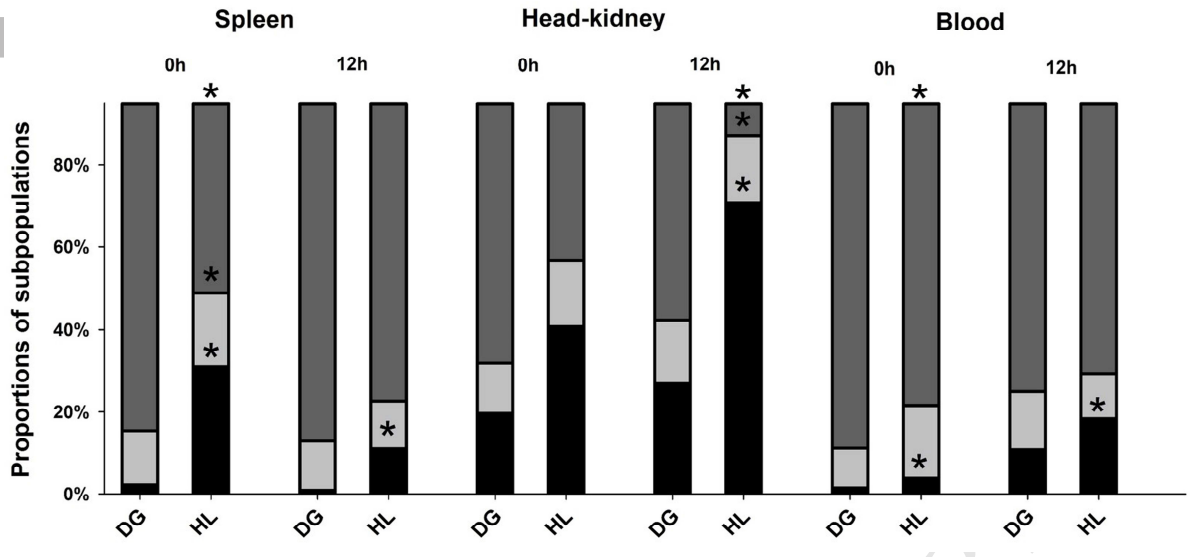
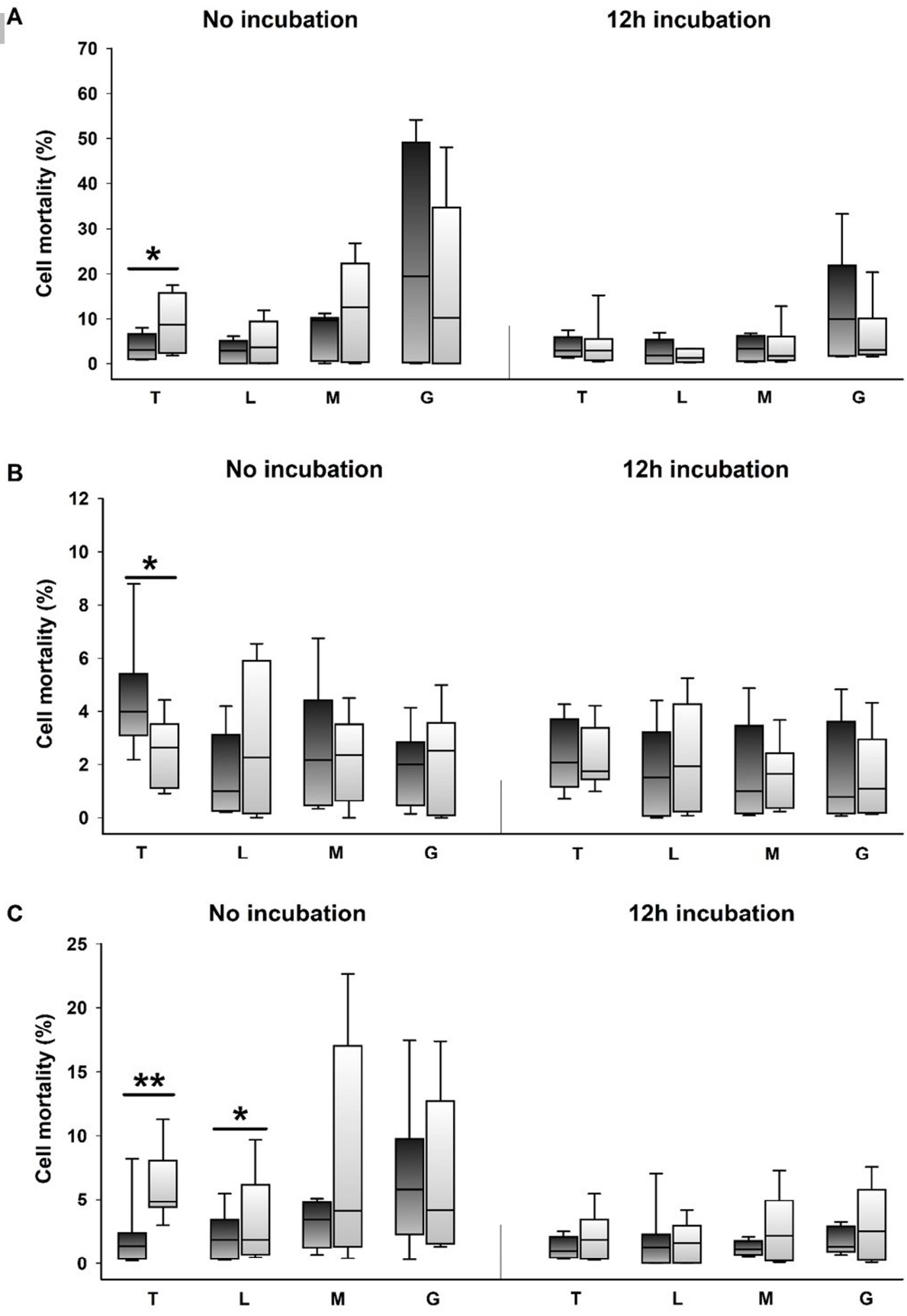

No incubation
12h incubation
Spleen
Head-kidney
Blood
Gate: P1 except (P2 in P1)
Gate: P1 except P2
SSC-A
FSC-A
583 138
538 138
2 000 000
3 700 000
Density gradient
Hypotonic lysis

Fig.6



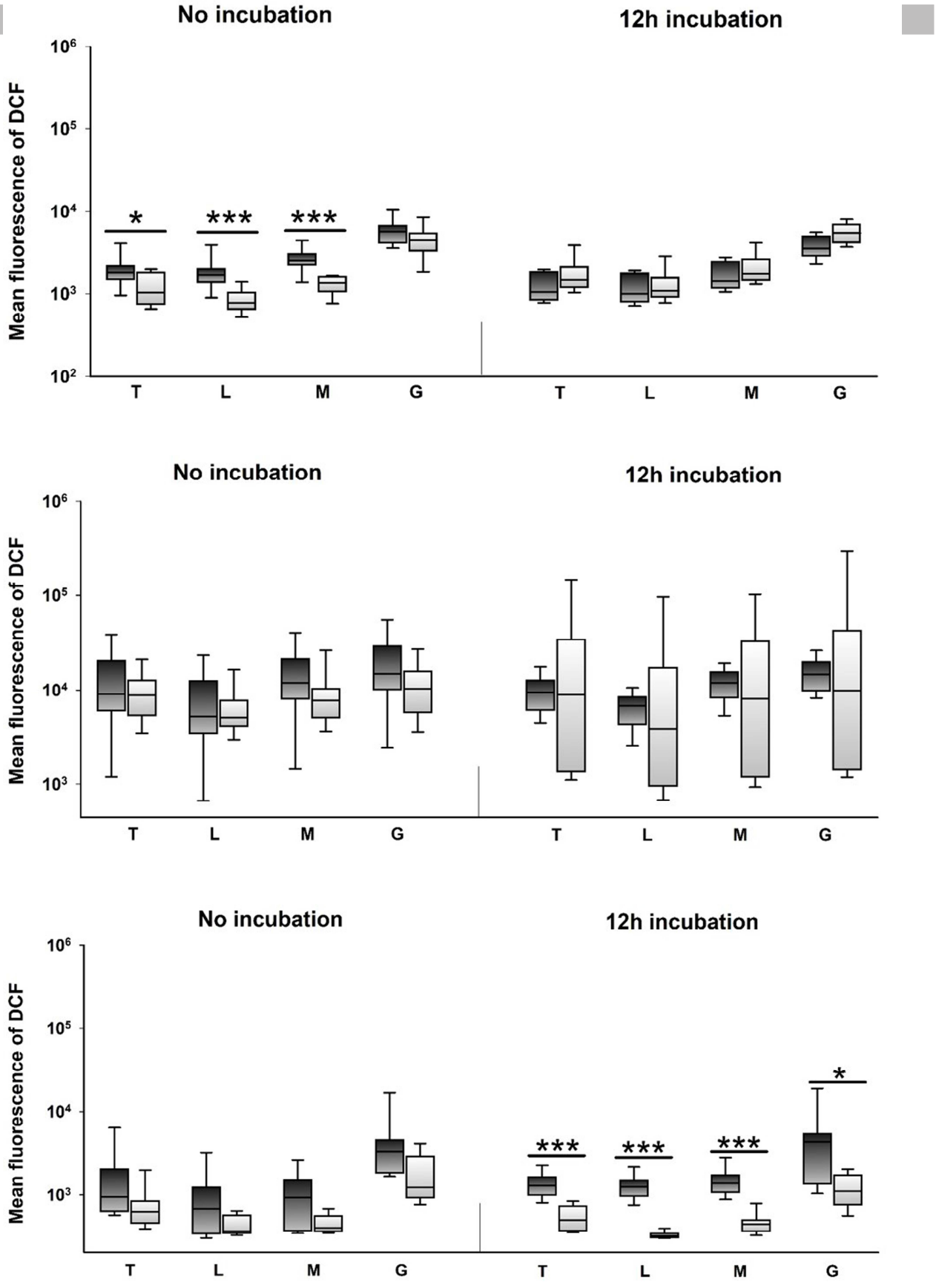
1066

1067 **Fig.7**



1068

1069 **Fig.8**



1070

1071 **Fig.9**

Highlights:

- **Leukocytes purified from spleen, head-kidney and blood of roach by hypotonic lysis shows heterophils enrichment in contrast to density gradient.**
- **Cellular responses used for evaluation of fish immune status were influenced by procedures used for leukocytes isolation.**
- **Spleen was the lymphoid tissue whose leukocytes were the lowest influenced by isolating procedure used.**