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Optimisation of the lymphocyte proliferation assay in rainbow trout (*Oncorhynchus mykiss*)

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Abstract: The lymphocyte proliferation assay is a valuable method used for the evaluation of the fish immune system. However, there are many variations and optimal results are not always obtained. Unification is necessary to ensure the comparability between different studies. The aim of this study was to optimise the lymphocyte proliferation assay in rainbow trout (*Oncorhynchus mykiss*). This goal included the determination of the optimal incubation length, serum type, incubation temperature, type of mitogen and its concentration, and anticoagulant. The peripheral blood and head kidney lymphocytes were isolated by density gradient centrifugation. Subsequently, the cells were incubated for 3–8 days with different mitogens (pokeweed mitogen 5, 10 and 50 µg/ml, concanavalin A 1, 10 and 20 µg/ml, phytohaemagglutinin 25, 50 and 100 µg/ml, lipopolysaccharide 1, 50 and 100 µg/ml). The use of the different serum types (foetal bovine serum, trout serum), incubation temperatures (10–20 °C) and anticoagulants (heparin, EDTA) was compared. Labelled thymidine was used to evaluate the assay. The best results were obtained after seven days of incubation at 15 °C with foetal bovine serum (FBS). The head kidney lymphocytes showed the highest proliferative response with 50 µg/ml phytohaemagglutinin. With the peripheral blood lymphocytes (heparin and EDTA), the best results were obtained with 50 µg/ml pokeweed mitogen. The highest proliferation levels were detected with heparinised blood. In conclusion, optimisation of this assay contributes to the improved assessment of the rainbow trout immune function.

Keywords: fish; mitogen; stimulation; thymidine; transformation

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Due to the rapid increase in the demand and production of fish meat, intensive aquaculture systems often deal with problems caused particularly by high stocking density and increased stress in fish. These conditions lead to various problems which are also reflected in the fish immune system – its ability to react can be examined by functional immunological assays (Kollner et al. 2002).

The lymphocyte proliferation assay was described by Nowell (1960). During the assay, lymphocytes are transformed into lymphoblasts after mitogenic stimulation and, subsequently, their proliferation activity is measured. Plant mitogens providing polyclonal activation of lymphocytes are most commonly used. Similarly to mammals, T lymphocytes are stimulated by phytohaemagglutinin and concanavalin, B lymphocytes are stimulated by lipopolysaccharide, and pokeweed mitogen stimulates both cell types, although primarily T blastogenesis was observed in rainbow trout (*Oncorhynchus mykiss*) by some authors (Janossy and Greaves 1971; Chilmonczyk 1978; Kehrer et al. 1998). The purity of the mitogens may differ and may be the cause of the different results in some cases. The activation and proliferation of leukocytes is an important part of every immune response (Rycyzyn et al. 1998). This test provides a wide range of utilisation possibilities considering that the lymphocyte proliferation can be influenced by many factors, including diseases, pollutants, rearing conditions and stress (Espelid et al. 1996; Wendelaar Bonga 1997). It has been commonly used in mammals (Divya et al. 2015; Kralova-Kovarikova et al. 2016), but in fish, there are many different methodologies for various species and optimal results are not always obtained. The LTT (Lymphocyte Transformation Test) can be applied to assess the effect of pesticides on the lymphocyte viability and proliferation (Shelley et al. 2012), and is also often used in other toxicological studies (Harford et al. 2007; Muller et al. 2009) as well as nutritional studies (Siwicki et al. 2003; Kowalska et al. 2017).

Different incubation conditions were used in the previous studies with rainbow trout lymphocytes. The incubation time usually varies from 3 to 5 days, the shorter period being more common (Tahir and Secombes 1995; Siwicki et al. 2003; Shelley et al. 2012). Incubation with foetal bovine serum (FBS) as well as trout serum was described (Verlhac et al. 1998; Siwicki et al. 2003; Hebert et al. 2008). Temperature ranged from 15 °C to 22 °C (Tahir and Secombes 1995; Lunden and Bylund 2000; Shelley et al. 2012). The usual mitogens are phytohaemagglutinin, concanavalin A and lipopolysaccharide (Flory and Bayne 1991; Siwicki et al. 2003), stimulation with pokeweed mitogen is less common (Ottinger and Kaattari 2000). In fish, mitogen concentrations vary from 1 µg/ml to 200 µg/ml (sometimes even 1000) (Chilmonczyk 1978; Wang et al. 1997; Kehrer et al. 1998). The most commonly used samples include peripheral blood, the head kidney and spleen (O'Halloran et al. 1998; Shelley et al. 2012).

There are many possibilities when it comes to the evaluation of the lymphocyte proliferative response. One of the traditional options is the use of methyl-^{3}H-thymidine (Yun et al. 2006; Harford et al. 2007; Muller et al. 2009). This method requires special equipment and involves work with radiolabelled substances, but it is sensitive and reliable. Another frequently applied evaluation method is the incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) and its measurement by an ELISA-based assay (van der Heijden et al. 1995; Gauthier et al. 2003). It has been mainly used in mammals (Walsh et al. 2005; Karami et al. 2016) and less often in birds (Finkelstein et al. 2007; Grasman et al. 2013). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay is another alternative (Mosmann 1983; Siwicki et al. 2003; Kowalska et al. 2017), as well as the evaluation by flow cytometry (Scharsack et al. 2001; Shelley et al. 2012). However, these assays were mostly optimised in mammals, so they are often unreliable and have limitations like the detection range and other technical issues (Gauthier et al. 2003). Therefore, the use of labelled thymidine can be a suitable option. Also, in contrast to using a commercial kit, an unlimited number of samples can be examined.

The aim of this work was to optimise the lymphocyte proliferation assay in rainbow trout. To achieve this goal, the optimal length of incubation, serum type, incubation temperature, type of mitogen and its concentration and anticoagulant had to be determined.

MATERIAL AND METHODS

Fish. An all-female population of rainbow trout (*Oncorhynchus mykiss*) was kept in an experimental

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recirculating system situated at Mendel University in Brno (Czech Republic). The fish (average weight 220.46 ± 121.11 g, total length 24.91 ± 3.53 cm) were stocked in four breeding tanks, each with a volume of approximately 1000 l. A Nexus 310 biofilter and a UV-C lamp were used for water filtration and disinfection. All fish were fed two times a day with a BioMar EFICO Enviro 920 feed (BioMar, Denmark).

The average water temperature was 17.5 °C, the oxygen saturation was 87.2% (8.15 mg/l), the pH was 7.2, $N\text{-}NH_4^+$ 0.39 mg/l, $N\text{-}NO_2^-$ 0.18 mg/l and Cl^- 145.83 mg/l.

The experiments were performed in accordance with the EU Directive 2010/63/EU for animal experiments. The Czech national regulations for animal experimentation were also followed (Act No. 246/1992 Coll.) and the experiments were approved by the Central Commission for Animal Welfare (MŠMT-19896/2015).

Sample collection. Every studied part of the test (the length of incubation, the serum, the temperature, the mitogens and the anticoagulants) was examined in a separate experiment (the mitogens in two), usually with eight (up to twelve) fish sampled. The data sample varies based on the numbers of the isolated cells. At first, some incubation parameters were selected based on pre-experiments. Blood (1 ml) was collected from the caudal vein into a heparinised (Zentiva, Czech Republic) syringe or into a syringe with EDTA (0.5M; ethylenediaminetetraacetic acid; Sigma-Aldrich, USA). The non-heparinised blood for the serum preparation was also obtained at this point. Consequently, the fish were humanely euthanised by a blow to the head, the samples of the head kidney were taken and placed into DPBS (Dulbecco's phosphate-buffered saline; Lonza, Switzerland). All samples were kept on ice and processed in less than two hours after collection. All the procedures described below were carried out in a laminar flow cabinet, under sterile conditions.

Isolation of lymphocytes. The head kidney samples were forced through a fine nylon mesh and washed by centrifugation in DPBS for 2 × 10 min at 450 *g*. The blood samples were diluted 1 : 2 by RPMI-1640 Medium (Sigma-Aldrich, USA). Subsequently, all samples were layered on Histopaque (1077 g/ml; Sigma-Aldrich, USA) and the mononuclear cells were isolated by density gradient centrifugation at 15 °C for 40 min at 800 *g*. The layer of mononuclear cells formed on the interface was collected and washed by centrifugation in DPBS for 10 min at 450 *g*. The isolated cells were counted by the BC-2800Vet haematology analyser (Mindray, China) and resuspended in L-15 Medium (Leibovitz, Sigma-Aldrich, USA) to the concentration of 2×10^5 cells/well.

Lymphocyte proliferation assay. The lymphocyte suspension and different mitogens were dispensed into 96-well plates (Techno Plastic Products, Switzerland) making a total reaction volume of 200 µl (2×10^5 cells). The heat inactivated (60 °C for 30 min) and sterile filtered foetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, UK) or the heat inactivated (44 °C for 20 min as described by Sakai (1981)) autologous or homologous trout serum was added to each well (10%). The mitogens comprised 5, 10 and 50 µg/ml pokeweed mitogen (PWM; Sigma-Aldrich, USA), 1, 10 and 20 µg/ml concanavalin A (ConA; Sigma-Aldrich, USA), 25, 50 and 100 µg/ml phytohaemagglutinin (PHA; Thermo Fisher Scientific, USA) and 1, 50 and 100 µg/ml lipopolysaccharide (LPS; prepared by phenol extraction from *Actinobacillus pleuropneumoniae*). During the preliminary experiments, two times higher than the highest and two times lower than the lowest concentration of each mitogen were used. All samples including the non-stimulated negative controls were run in triplicates. The lymphocytes were placed in an incubator (Memmert, Germany) at 10 °C, 15 °C or 20 °C. After 3–8 days, 50 µl of ^{3}H-thymidine (5 µCi/ml; American Radiolabeled Chemicals, USA) was added and incorporated into the newly formed DNA of the proliferating lymphocytes. After 20 h, the cells were harvested by the FilterMate Harvester (Packard, USA) and the proliferation activity was measured as counts per minute (cpm) by the TopCount NXT liquid scintillation counter (Packard, USA). The stimulation index (SI; the mean ratio of the stimulated and non-stimulated lymphocytes) was calculated.

Statistical analyses. For the statistical analyses, the Shapiro-Wilk test was used to determine if the data are normally distributed. Subsequently, the Mann-Whitney *U* test or Student's *t*-test, and the multi-sample median test or Tukey's HSD (Honestly Significant Difference) test was performed, depending on the parametricity of the data. The means and standard errors of the means (SEM) were calculated; $P < 0.05$ was considered significant.

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RESULTS

Length of incubation

The optimal length of incubation was investigated. Due to the different numbers of the isolated cells from each fish, the cells were primarily incubated for 5–7 days, resulting in a lower data sample when a longer time period is included in the statistics evaluation. The incubation of the head kidney lymphocytes was carried out for 8 days (with the cell proliferation measured during day 3–8) with pokeweed mitogen (10 µg/ml) and foetal bovine serum at 15 °C (the mitogen, temperature and serum were selected based on the pre-experiments). The best results were obtained when incubated for 7 days (SI 24.72). There was a significant difference between the 3- and 6-day incubation ($P < 0.05$; Figure 1A). With the individual statistical analysis of the 6- and 7-day incubation (with a larger data sample), higher values were still obtained after 7 days (SI 32.51); however, the differences were not statistically significant (Figure 2).

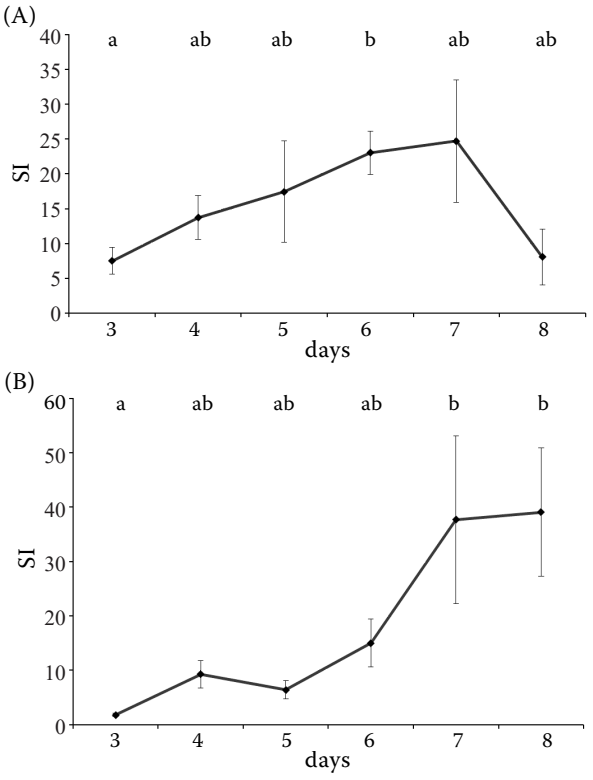

Figure 1. The effect of the incubation time (3–8 days) on the proliferation of the head kidney lymphocytes (**A**) and the peripheral blood lymphocytes collected using heparin (**B**). The cells were incubated at 15 °C with pokeweed mitogen (10 µg/ml) and foetal bovine serum. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM, A: $n = 4$; B: $n = 5$) with the different superscript letters are significantly different ($P < 0.05$)

With the peripheral blood lymphocytes (collected using heparin), the highest proliferation levels were detected after 8 days of incubation (SI 47.04). The cells were incubated for 8 days at 15 °C with pokeweed mitogen (10 µg/ml) and FBS. A significant difference was detected between 3 days and 7 + 8 days of incubation ($P < 0.05$; Figure 1B). However, when 5–8 days of incubation were analysed separately (with a larger data sample), the highest values were obtained at 7 days (SI 61.23; Figure 3).

Serum

The optimal serum type was investigated – incubation with FBS, autologous and homologous trout serum was compared. The head kidney lymphocytes were incubated for 7 days at 15 °C with 100 µg/ml

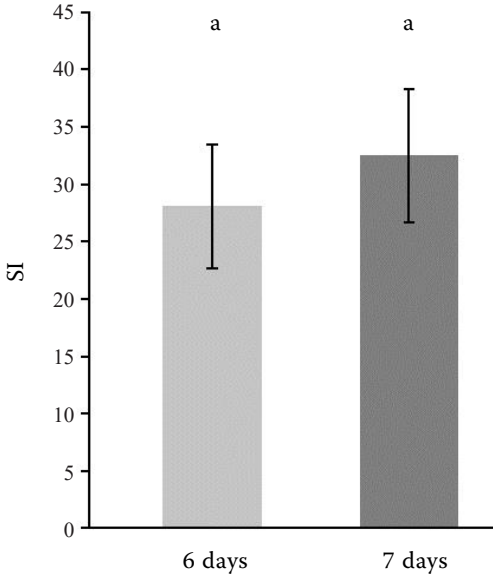

Figure 2. The effect of the incubation time (6–7 days) on the proliferation of the head kidney lymphocytes. The cells were incubated at 15 °C with pokeweed mitogen (10 µg/ml) and foetal bovine serum. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values are the means ± SEM ($n = 12$), no significant differences were detected

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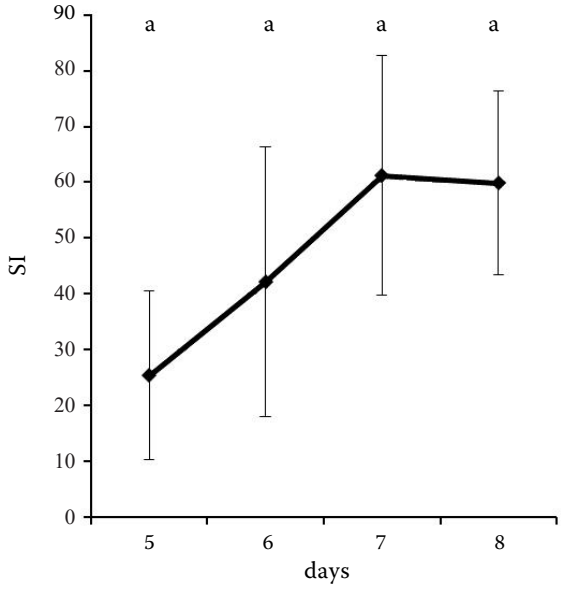

Figure 3. The effect of the incubation time (5–8 days) on the proliferation of the peripheral blood lymphocytes collected using heparin. The cells were incubated at 15 °C with pokeweed mitogen (10 µg/ml) and foetal bovine serum. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values are the means ± SEM (n = 7), no significant differences were detected

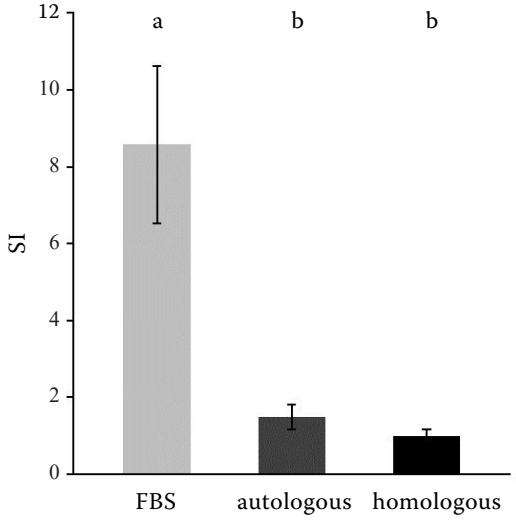

Figure 4. The effect of the different sera (foetal bovine serum – FBS, autologous or homologous trout serum) on the proliferation of the head kidney lymphocytes. The cells were incubated for 7 days at 15 °C with phytohaemagglutinin (100 µg/ml) and the different sera. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM) with the different superscript letters are significantly different (n = 6; P < 0.01)

phytohaemagglutinin (the selected temperature and mitogen were based on the pre-experiments); FBS showed significantly higher proliferation levels than trout serum (P < 0.01; Figure 4).

Incubation temperature

The optimal incubation temperature was determined. The head kidney lymphocytes were incubated at 10 °C, 15 °C, and 20 °C for 7 days with FBS and 100 µg/ml phytohaemagglutinin (the selected mitogen was based on the pre-experiments). There were no significant differences between the different incubation temperatures (Figure 5A).

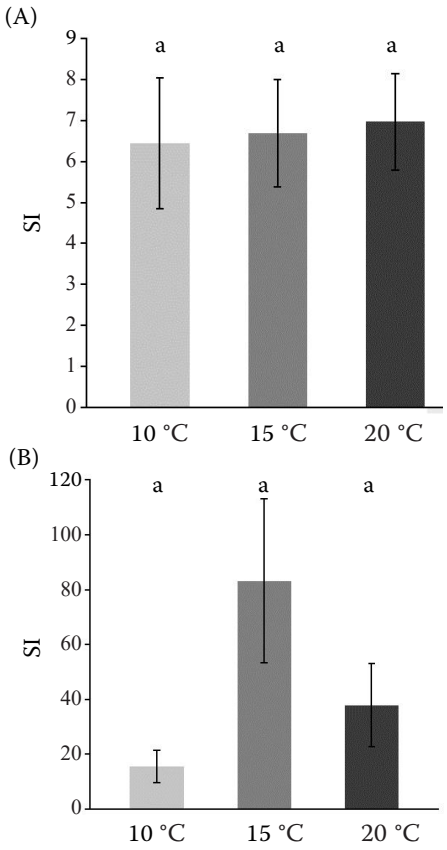

Figure 5. The effect of the incubation temperature (10, 15 and 20 °C) on the lymphocyte proliferation. The head kidney lymphocytes (**A**) were incubated for 7 days with foetal bovine serum and phytohaemagglutinin (100 µg/ml), the peripheral blood lymphocytes collected using heparin (**B**) were incubated with pokeweed mitogen (50 µg/ml). The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values are the means ± SEM (n = 8), no significant differences were detected

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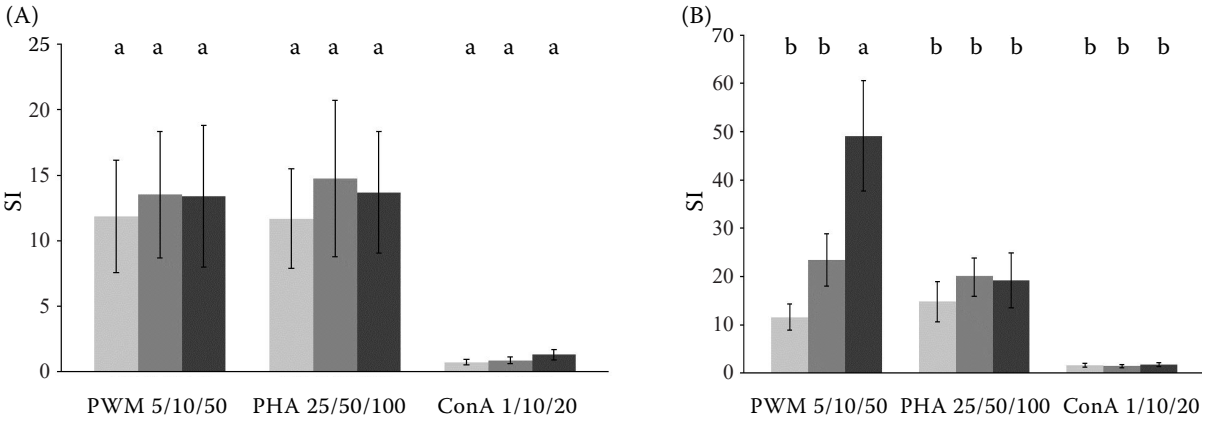

Figure 6. The effect of the different mitogens on the proliferation of the head kidney lymphocytes (**A**) and the peripheral blood lymphocytes collected using heparin (**B**) – pokeweed mitogen (PWM; 5, 10, 50 µg/ml), phytohaemagglutinin (PHA; 25, 50, 100 µg/ml) and concanavalin A (ConA; 1, 10, 20 µg/ml). The cells were incubated for 7 days at 15 °C with foetal bovine serum and the different mitogens. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM, A: $n = 6$; B: $n = 8$) with the different superscript letters are significantly different (A: no significant differences were detected; B: $P < 0.05$)

The peripheral blood lymphocytes collected with the use of heparin were incubated for 7 days with FBS and pokeweed mitogen (50 µg/ml). The highest proliferation levels were measured with the incubation carried out at 15 °C (SI 83.16; Figure 5B).

Mitogens and their concentrations

Subsequently, the optimal mitogens and their concentrations were determined. The cells were incubated for 7 days at 15 °C with FBS and the different mitogens (2.4.). The head kidney lymphocytes

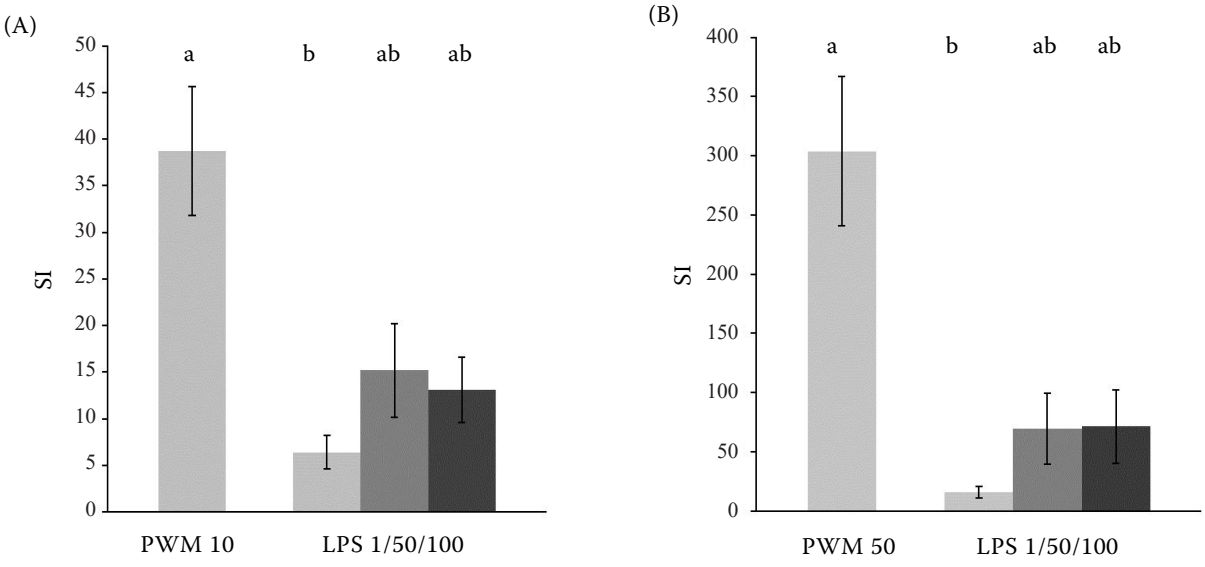

Figure 7. The effect of the different mitogens on the proliferation of the head kidney lymphocytes (**A**) – pokeweed mitogen (PWM; 10 µg/ml) and lipopolysaccharide (LPS; 1, 50, 100 µg/ml). The effect of the different mitogens on the proliferation of the peripheral blood lymphocytes collected using heparin (**B**) – pokeweed mitogen (PWM; 50 µg/ml) and lipopolysaccharide (LPS; 1, 50, 100 µg/ml). The cells were incubated for 7 days at 15 °C with foetal bovine serum and the different mitogens. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM, $n = 8$) with the different superscript letters are significantly different (A: $P < 0.05$; B: $P < 0.01$)

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showed very low results with concanavalin A (no stimulation was observed) compared to pokeweed mitogen and phytohaemagglutinin (Figure 6A). The highest values were detected with 50 µg/ml phytohaemagglutinin. Pokeweed mitogen (10 µg/ml) also provided significantly higher proliferation levels than 1 µg/ml lipopolysaccharide ($P < 0.05$; Figure 7A).

The peripheral blood lymphocytes isolated from heparinised blood showed the best results with 50 µg/ml pokeweed mitogen (SI 49.09), with a significant difference compared to its other concentrations, phytohaemagglutinin and concanavalin A ($P < 0.05$). With concanavalin A, almost no stimulation was observed (Figure 6B). In comparison with 1 µg/ml lipopolysaccharide, 50 µg/ml pokeweed mitogen provided a significantly higher proliferative response (11 536.88 cpm/SI 303.68; $P < 0.01$; Figure 7B).

Similarly to the heparinised blood, the peripheral blood lymphocytes collected with the use of EDTA (incubated for 6 days – the optimal incubation time based on the pre-experiments) were well stimulated by 50 µg/ml pokeweed mitogen (SI 44.25), with a significant difference compared to 50 µg/ml phytohaemagglutinin ($P < 0.01$), where no stimulation was observed (Figure 8).

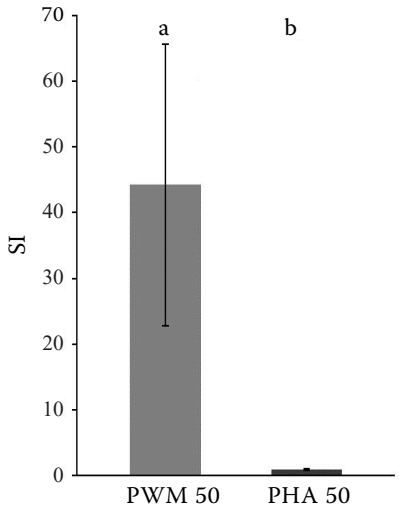

Figure 8. The effect of the different mitogens on the proliferation of the peripheral blood lymphocytes collected using EDTA – pokeweed mitogen (PWM; 50 µg/ml) and phytohaemagglutinin (PHA; 50 µg/ml). The cells were incubated for 6 days at 15 °C with foetal bovine serum and the different mitogens. The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM) with the different superscript letters are significantly different ($n = 10$; $P < 0.01$)

Anticoagulants

The proliferation activity of the blood samples collected using heparin or EDTA was compared. The cells isolated from peripheral blood were incubated for 7 days at 15 °C with foetal bovine serum and pokeweed mitogen (50 µg/ml). The heparinised blood significantly showed the best results (SI 278.08; $P < 0.01$), approximately 6 times higher than those detected with the use of EDTA (Figure 9).

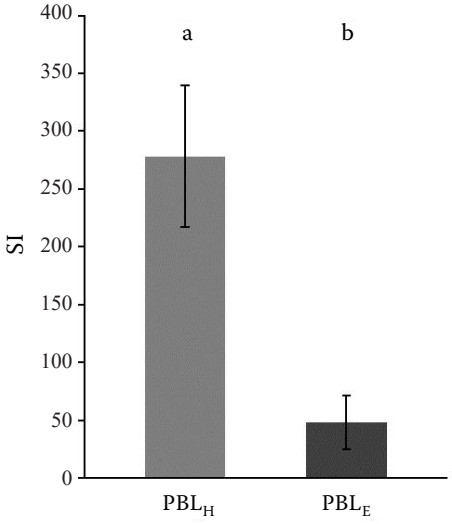

Figure 9. The effect of the different anticoagulants (heparin – PBL_H or EDTA – PBL_E) on the proliferation of the peripheral blood lymphocytes. The peripheral blood lymphocytes were incubated for 7 days at 15 °C with foetal bovine serum and pokeweed mitogen (50 µg/ml). The stimulation index (SI) is the mean ratio of the stimulated and non-stimulated lymphocytes (measured as counts per minute). The values (means ± SEM) with the different superscript letters are significantly different ($n = 9$; $P < 0.01$)

DISCUSSION

In other studies, a shorter incubation period (3–4 days) has usually been used (Chilmonczyk 1978; Kehrer et al. 1998; Gauthier et al. 2003; Siwicki et al. 2003; Muller et al. 2009; Shelley et al. 2012). In mammals, lymphocytes are also generally incubated for 2–4 days (Yaqoob et al. 1994; Kearns et al. 1999; Yun et al. 2006; Divya et al. 2015). However, our values were usually higher than those obtained in the studies using shorter (suboptimal) incuba-

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tion periods (Chilmonczyk 1978; Flory and Bayne 1991; Tahir and Secombes 1995; Lin et al. 1999; Gauthier et al. 2003; Leonardi and Klempau 2003; Hebert et al. 2008). Similar observations to ours were described by DeKoning and Kaattari (1991) and Reitan and Thuvander (1991). In their studies, longer incubation periods (5–6 days) provided higher stimulation levels.

As for the mitogens, the best results were acquired with pokeweed mitogen (11 536.88 cpm/SI 303.68). These values are comparable to the ones observed in mammals (Yaqoob et al. 1994; Wagner et al. 1999). Since pokeweed mitogen stimulates both B and T lymphocytes, the highest proliferation activity was expected. In our study, the T cell mitogen phytohaemagglutinin showed better results than the B cell mitogen lipopolysaccharide. This observation indicates that, in rainbow trout, T cells are the predominant leucocyte population stimulated by pokeweed mitogen, as observed by other authors (Kehrer et al. 1998). The use of concanavalin A resulted in a very low lymphocyte stimulation compared to the other mitogens. Different studies obtained similar proliferative responses using this mitogen (Gauthier et al. 2003; Leonardi and Klempau 2003). In contrast to our findings, Reitan and Thuvander (1991) observed no mitogenic effect of higher concentrations of PHA on peripheral blood lymphocytes. According to Etlinger et al. (1976), head kidney cells could be stimulated by LPS but not by ConA. The differences between the lymphocyte mitogenesis detected in the peripheral blood and head kidney lymphocytes were also described by Daly et al. (1995). The lymphocytes collected with the use of EDTA (inhibitor of cell activity) showed no proliferation with phytohaemagglutinin – the effect which has also been observed by other authors (Alford 1970). Generally, high concentrations of mitogens were usually more effective than the low concentrations in our study.

No stimulation was observed with autologous or homologous serum, although DeKoning and Kaattari (1991) describe different findings with homologous rainbow trout plasma. However, the values detected in the studies using homologous serum (Chilmonczyk 1978) were markedly lower compared to ours. Therefore, FBS (commonly used in mammals) seems to provide better proliferation conditions for the rainbow trout lymphocytes. It was used in many studies (Tillitt et al. 1988; Kehrer et al. 1998; Gauthier et al. 2003; Muller et al. 2009; Shelley et al. 2012), although some authors preferred autologous or homologous serum (Daly et al. 1995; Rycyzyn et al. 1998).

The most commonly used incubation temperature is 15–20 °C for rainbow trout lymphocytes (Kehrer et al. 1998; Gauthier et al. 2003; Muller et al. 2009; Shelley et al. 2012). Similarly, our data yielded the best results at 15 °C, with a decrease in the proliferation activity at 10 °C. Some authors use even higher incubation temperatures in rainbow trout (Chilmonczyk 1978; Siwicki et al. 1996; Verlhac et al. 1998; Lunden and Bylund 2000; Siwicki et al. 2003; Hebert et al. 2008). Because temperature is one of the most important factors in fish, its maintenance can be crucial (Bly and Clem 1992; Hardie et al. 1994).

The samples most commonly used in the lymphocyte proliferation assay comprise peripheral blood (van der Heijden et al. 1995; Kehrer et al. 1998; Rycyzyn et al. 1998; Gauthier et al. 2003; Shelley et al. 2012) and the head kidney (Gauthier et al. 2003; Siwicki et al. 2003; Kowalska et al. 2017). As the main haematopoietic organ in teleosts, the head kidney shows some proliferation activity even in the non-stimulated cells (Gauthier et al. 2003), consequently providing results similar to EDTA (cell activity inhibitor). In our experiment, the values (SI 278.08) obtained with the heparinised blood were significantly higher compared to most other studies (Chilmonczyk 1978; Tillitt et al. 1988).

The lymphocyte proliferation assay is a reliable method of examining the fish immune function. However, there are many variables involved and various authors have used different incubation lengths, temperatures, serum types and types of mitogens. Different results may be obtained due to the various conditions used during this assay. Therefore, unification of this method is essential to ensure comparability. In rainbow trout (*Oncorhynchus mykiss*), a longer incubation period (7 days) is necessary to obtain sufficient proliferation levels. Incubation at 15 °C and the use of FBS instead of trout serum is advisable. Examination of both peripheral blood cells and head kidney cells is appropriate to acquire a complex view of the immune response. Use of heparin instead of EDTA as anticoagulant is highly recommended. Based on our results, 50 µg/ml phytohaemagglutinin is the optimal T cell mitogen (in contrast to concanavalin A), 50–100 µg/ml lipopolysaccharide stimulates B cells and stimula-

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tion of both cell types is best provided by 50 µg/ml pokeweed mitogen. Due to the different cell representation in each sample, the peripheral blood lymphocytes are best stimulated by 50 µg/ml pokeweed mitogen while the head kidney lymphocytes show the highest proliferation levels with 50 µg/ml phytohaemagglutinin. Optimisation of this assay improves its utilisation in determining the fish health.

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