Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 10	Fasc. 2	143-152	2002

APPLICATION OF ANTI-STRESS PRODUCTS IN AQUACULTURE: INFLUENCE OF PROPISCIN ON THE EFFECTIVENESS OF AN ANTI-*YERSINIA RUCKERI* VACCINE IN RAINBOW TROUT *ONCORHYNCHUS MYKISS* (WAL.)

Andrzej K. Siwicki, ***, Marc Morand**, Krzysztof Kazuń*, Nicolas Keck**, Edward Głąbski*, Joanna Małaczewska****

*The Stanisław Sakowicz Inland Fisheries Institute in Olsztyn, Poland
**Laboratoire Departemental d' Analyses, Conseil General du Jura, France
***Department of Microbiology and Clinical Immunology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland

ABSTRACT. Inducing protection against bacterial diseases in fish culture through the application of specific vaccines is one of the most important prevention methods. However, some immunization techniques actually used under hatchery conditions are not as effective as they should be. Therefore, current research is focusing on how to improve the potency and efficacy of the antigens and how to optimally activate the immune response. In our preliminary study, we determined the influence of the anaesthetic Propiscin (IFI, Poland) on the effectiveness of a vaccine applied by intraperitoneal injection or immersion to rainbow trout *(Oncorhynchus mykiss).* The fish were vaccinated *via* injection or immersion with an anti-*Yersinia ruckeri* vaccine (Sanofi, France) under Propiscin-induced anaesthesia or without it. On day 21 after vaccination, all the fish groups were challenged with live *Y. ruckeri* bacteria (0.2 ml of bacteria at 1×10^6 ml^{-1}). Control groups (only anaesthetized and injected PBS or immersed) were infected but not immunized by vaccine. The blood and pronephros were separated before vaccination (10 fish), 21 days after vaccination (10 fish) and 21 days after the challenge (10 fish). ELISPOT assays for the quantification of total immunoglobulin secreting cell (ISC) and specific antibody secreting cell levels (ASC) were used. The total Ig levels in the serum were also measured by spectrophotometric assay and the titers of anti-*Y. ruckeri* antibody were measured by the micro-agglutination method. Mortalities were collected and tabulated and the presence of pathogen was confirmed by isolation from fish kidneys. The results of this study showed that Propiscin applied at vaccination time increased the nonspecific and specific immune response and protective effectiveness of vaccine after the challenge with pathogenic bacteria. The highest level of total ISC and specific ASC in fish vaccinated by immersion and injections under anaesthesia were observed. The highest total Ig levels and titers of specific antibody in fish vaccinated under anaesthesia were also observed. Cumulative mortality rates were lowest in the fish vaccinated by injection (5%) and immersion (10%) under anaesthesia as compared with fish vaccinated by injection (20%) and immersion (35%) without anaesthesia. Cumulative mortality in the control, non-vaccinated group of fish was 80%.

Key words: PROPISCIN, RAINBOW TROUT (*ONCORHYNCHUS MYKISS*), ANTI-*Y. RUCKERI* VACCINE, IMMUNE RESPONSE

INTRODUCTION

Fish exposed to pollutants, contaminants, intensive production stress, or given drug treatments to prevent diseases may exhibit a suppressed nonspecific defense mechanism and immune response, which renders them less able to protect themselves against diseases (Dunier and Siwicki 1994a). Many papers present the direct influence of environmental contamination or polyethiological stress (manipulation, transport) on the fish immune system and demonstrate that chemical and physical stress has an immunosuppressive influence on both humoral and cellular immune functions and, consequently, on physiological condition and disease resistance (Ellis 1988, Dunier and Siwicki 1994b, Studnicka et al. 2000).

The protective effects of vaccination are determined by the immunological condition of the immunized fish. If polyethiological stress or pollutants suppress defense mechanisms, the immune response after vaccination will be lower and not effective for long-term protection against a specific antigen. Also, vaccination methods (manipulation stress, concentration of antigen in immersion vaccine) constitute a stress situation that stimulates strong mucus secretion which restricts the contact of the vaccine antigen with immunocompetent cells and increases the cellular and humoral immune response on the vaccine antigen (Smitt et al. 1979, Ellis 1988, Anderson 1992, Siwicki et al. 1998a,b, Studnicka et al. 2000).

Anaesthetics are one of the most important products applied in aquaculture. They have been used extensively in intensive fish culture to reduce the effects of stress on fish and to lower mortality after handling and transporting large stocks of fish. These agents not only allow handling for breeding or medical purposes to be achieved quickly and simply, but they also decrease the effects of stress in the fish during these procedures (Jeney et al. 1986, Trzebiatowski et al. 1996, Veenstra et al. 1987). One of the most popular anaesthetic is Propiscin (IFI, Poland), which induces a short period of general anaesthesia that lasts about 30 minutes (Kazuń and Siwicki 2001).

The idea of our study is to develop the possibility of reducing stress factors at vaccination by applying an anti-stress product to increase the effectiveness of the vaccine. In the present study, we examined the influence of the anaesthetic Propiscin (IFI, Poland) on the effectiveness of a vaccine applied by intraperitoneal injection or immersion in rainbow trout *Oncorhynchus mykiss* (Wal.).

MATERIAL AND METHODS

FISH AND EXPERIMENTAL DESIGN

For this experimental study, 300 healthy rainbow trout, weighing 30-40 g were purchased from a local fish farm. The fish were maintained in six, 500-liter tanks with a continuous flow of spring water at a temperature of 12 ± 1°C. The fish were fed daily with commercial pellets (BIOMAR) at 1% of their body weight. After one week of adaptation, the fish from four groups were vaccinated *via* intraperitoneal injection or immersion with a commercial anti-*Yersinia ruckeri* vaccine (VACCIN ANTI-YERSINIOSE, Sanofi Sante Animale, France) under anaesthesia with Propiscin (IFI, Poland) or without it. On day 21 after vaccination, all of the fish groups were challenged with live *Yersinia ruckeri* bacteria (0.2 ml of bacteria at 1×10^6 ml^{-1}). The control groups (only anaesthetized and injected PBS or immersed) were infected but not immunized by vaccine. The blood and pronephros were separated before vaccination (10 fish), 21 days after vaccination (10 fish) and 21 days after the challenge (10 fish).

ASSAY PROCEDURES

Blood and pronephros from each of the experimental and control groups were removed. The single cell suspensions from pronephros were obtained by teasing the tissues in a medium (RPMI-1640, Sigma) through a steel mesh. Cell suspensions were purified on a Histopaque (density 1.077, Sigma) gradient. Counts of living cells from spleen and pronephros were made with trypan blue using a haemocytometer after three washes in the medium (RPMI 1640, Sigma).

ELISPOT assays for the quantification of the total immunoglobulin secreting cells (ISC) and specific antibody secreting cells level (ASC) were used according to the protocol presented in Siwicki and Dunier (1993). Multiscreen-HA 96 well filtration plates of cellulose esters (0.45 μm, Millipore, Bedford, MA, USA) for isolation and identification spot-forming cells per 10^6 leucocytes from pronephros were used. The assays were done in quadruplicate for each fish.

The total Ig levels in the serum of each fish were measured by spectrophotometric assay, according to the method presented by Anderson and Siwicki (1994b).

The titers of the specific anti-*Y. ruckeri* antibody of each fish sera assayed in ELISPOT were simultaneously determined using the micro-agglutination method described by Cossarini-Dunier (1985).

Mortalities were collected and tabulated and the presence of the pathogen was confirmed by isolation from fish kidneys.

For statistical analysis, means and standard deviations for all values were calculated, and Student's t-test was used to determine differences between two or more groups. The significance level used was $P < 0.05$.

RESULTS AND DISCUSSION

In this study we determined the influence of Propiscin-induced anaesthesia on the nonspecific and specific immune response and protection against *Yersinia ruckeri* after immunization of rainbow trout with an anti-*Yersinia ruckeri* vaccine applied by immersion or injection. Fig. 1 summarizes the effects of Propiscin–induced anaesthesia on the immunoglobulin secreting cells (ISC) of rainbow trout vaccinated with the anti-*Yersinia ruckeri* vaccine by immersion or injection (day 21 after vaccination) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) in comparison with fish which were only vaccinated and control fish. Fig. 2 summarizes the specific

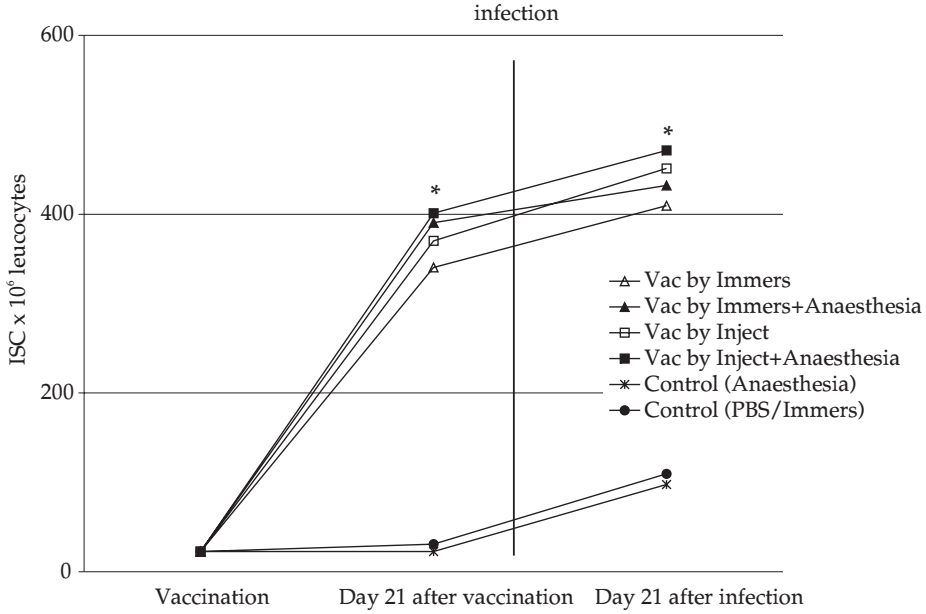

Fig. 1. Influence of Propiscin-induced anaesthesia on the immunoglobulin secreting cells (ISC) of rainbow trout vaccinated with anti-*Y. ruckeri* vaccine by immersion or injection (day 21) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) compared to fish that were only vaccinated and control fish (mean ± SD ≤ 5% of mean, n=10). * - marked values are statistically different vs. appropriate control group ($P < 0.05$)

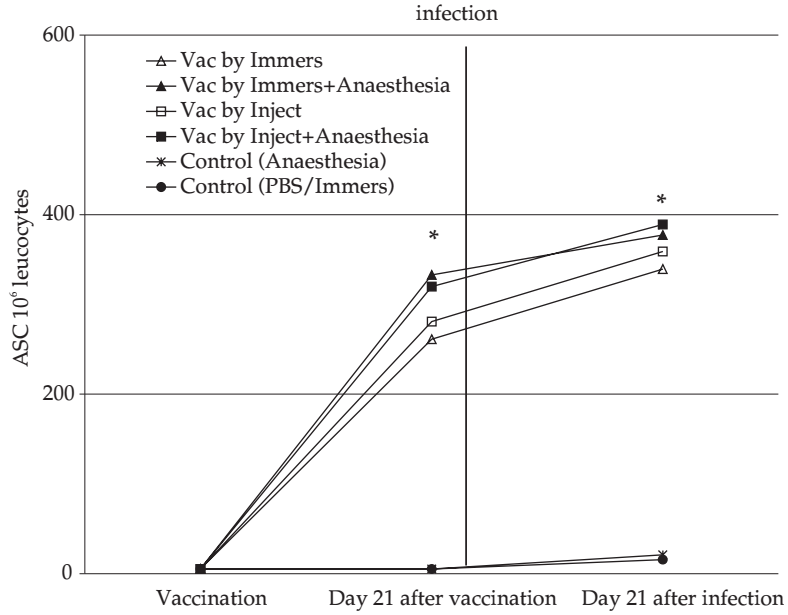

Fig. 2. Influence of Propiscin-induced anaesthesia on the antibody secreting cells (ASC) of rainbow trout vaccinated with anti-*Y. ruckeri* vaccine by immersion or injection (day 21) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) compared to fish that were only vaccinated and control fish (mean ± SD ≤ 5% of mean, n=10). * - marked values are statistically different vs. appropriate control group ($P < 0.05$)

antibody secreting cells (ASC) of rainbow trout vaccinated with the anti-*Yersinia ruckeri* vaccine by immersion or injection (day 21 after vaccination) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) in comparison with fish which were only vaccinated and control fish. In all the vaccinated groups of fish, the total ISC and specific ASC levels significantly increased in comparison with the control fish (non-vaccinated). However, different levels (statistically significant $P < 0.05$) in total ISC and specific ASC were observed between fish vaccinated under Propiscin-induced anaesthesia in comparison with fish vaccinated without anaesthesia. The highest levels of total ISC and specific ASC were observed in fish vaccinated by immersion and injection under anaesthesia.

Fig. 3 summarizes the effects of anaesthesia by Propiscin on the total Ig levels in the serum of rainbow trout vaccinated with the anti-*Yersinia ruckeri* vaccine by immersion or injection (day 21 after vaccination) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) in comparison with fish that were only vaccinated and control fish. Fig. 4 summarizes the specific antibody titers of rainbow trout

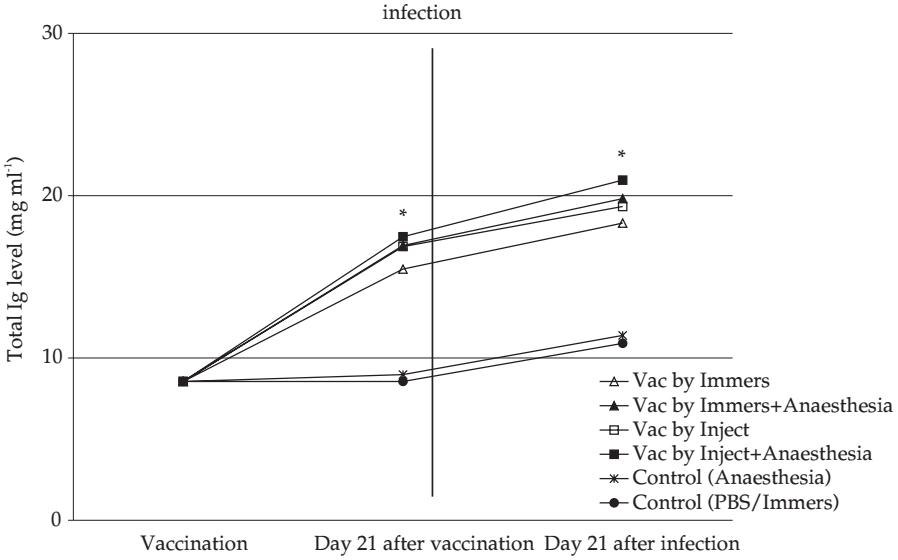

Fig. 3. Influence of Propiscin-induced anaesthesia on the total Ig level in serum of rainbow trout vaccinated with anti-*Y. ruckeri* vaccine by immersion or injection (day 21) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) compared to fish that were only vaccinated and control fish (mean ± SD ≤ 5% of mean, n=10). * - marked values are statistically different vs. appropriate control group ($P < 0.05$)

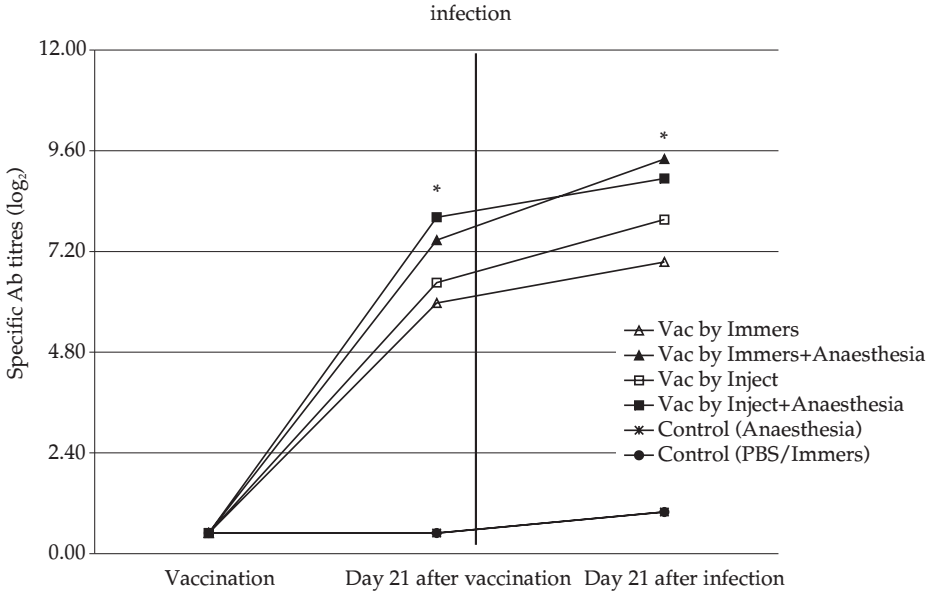

Fig. 4. Influence of Propiscin-induced anaesthesia on the specific antibody titres in serum of rainbow trout vaccinated with anti-*Y. ruckeri* vaccine by immersion or injection (day 21) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) compared to fish that were only vaccinated and control fish (mean ± SD ≤ 5% of mean, n=10). * - marked values are statistically different vs. appropriate control group ($P < 0.05$)

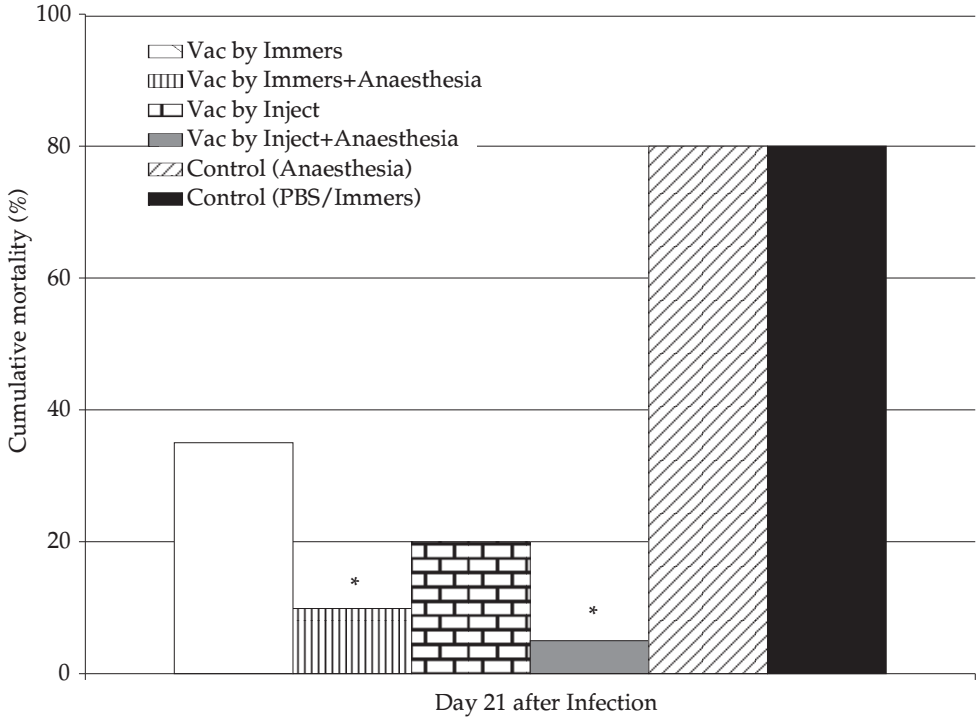

Fig. 5. Influence of Propiscin-induced anaesthesia on the percentage of cumulative mortality of rainbow trout vaccinated with anti-*Y. ruckeri* vaccine by immersion or injection and experimentally infected with *Yersinia ruckeri* compared to fish that were only vaccinated and control fish. * - marked values are statistically different vs. appropriate control group ($P < 0.05$)

vaccinated with anti-*Yersinia ruckeri* vaccine by immersion or injection (day 21 after vaccination) and experimentally infected with *Yersinia ruckeri* (day 21 after infection) in comparison with fish that were only vaccinated and control fish. In all the vaccinated groups of fish the total Ig levels and specific antibody titers in the serum significantly increased ($P < 0.05$) in comparison with the control fish (non-vaccinated). However, different levels (statistically significant $P < 0.05$) in total Ig levels and specific antibody titers in the serum were observed between fish vaccinated under Propiscin-induced anaesthesia in comparison with fish that were only vaccinated (without anesthesia). The highest levels of total ISC and specific ASC in fish vaccinated by immersion and injection under anaesthesia were observed.

The vulnerability of rainbow trout to the *Yersinia ruckeri* challenge and the resultant protection presented by the percentage of cumulative mortality of rainbow trout vaccinated with the anti-*Yersinia ruckeri* vaccine by immersion or injection and experi-

mentally infected with *Yersinia ruckeri* in comparison with fish that were only vaccinated and control fish is presented in Fig. 5. Cumulative mortality rates were lowest in the fish vaccinated by injection (5%) and immersion (10%) under anaesthesia in comparison with fish that were vaccinated by injection (20%) and immersion (35%) without anaesthesia. The cumulative mortality in the non-vaccinated control group of fish was 80%.

The application of vaccines has been an important method in preventing bacterial diseases of fish. When actually applied under hatchery conditions, some vaccines and immunization techniques are not as effective as they should be. Therefore, current research focuses on improving the potency and efficacy of antigens and determining how to optimally activate the nonspecific defence mechanisms and specific cellular and humoral immune response. One of the most frequent uncertainties regarding the use of vaccines is effective, long-term protection. The use of adjuvants and immunostimulants in fish culture offers a wide range of attractive methods for inducing and modulating protection against diseases (Ellis 1988, Nikl et al. 1991, Anderson 1992, Anderson and Siwicki 1994a, Siwicki et al. 1998a, b, Siwicki et al. 2001).

In our study we examined the influence of Propiscin-induced anaesthesia on the effectiveness of vaccines in fish. This experiment indicated the positive effects of anaesthesia on the nonspecific and specific immune response of rainbow trout induced with anti-*Yersinia ruckeri* vaccine applied by immersion or injection. The anaesthesia probably eliminated the negative reaction of mucous secretion and functional disorders induced by polyethiological stress. During the immersion method of immunization, the fish under anaesthesia has better contact with the antigen and more time to absorb it which induced the cell-mediated reaction. Antigen injection under anaesthesia eliminated stress and led to a more effective specific immune response. The initiation of the specific immune response is controlled by mononuclear phagocytic cells (antigen presenting cells – APC) involved in particle or antigen uptake. The information concerning what to make an antibody against is transferred to the B cells. Complex receptors on the cells, such as the major histocompatibility complexes (MHCs) that guide the selection of the antibody are assisted by messenger chemical molecules, cytokines and interleukins that are involved in the expression and control of antibody secretion.

The results of our preliminary studies showed that Propiscin applied at vaccination time increased the nonspecific and specific immune response and protective effectiveness of vaccine after a pathogenic bacteria challenge. Future studies will

include determining the optimal time of Propiscin-induced anaesthesia at the time of immunization and a protocol for vaccination by immersion with an anti-stress product to maximize protection after vaccination.

REFERENCES

Anderson D.P. 1992 – Immunostimulants, adjuvants, and vaccine carriers in fish: application to aquaculture – Annu. Rev. Fish Dis. 2: 281-307.

Anderson D.P., Siwicki A.K. 1994a – Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion – Prog. Fish - Cult. 56: 258-261.

Anderson D.P., Siwicki A.K. 1994b – Simplified assays for measuring nonspecific defense mechanisms in fish – Fish Health Section. Fisheries Soc. Meeting, Seattle, WA, 26 pp.

Cossarini-Dunier M. 1985 – Indirect enzyme-linked immunosorbent assay (ELISA) to titrate rainbow trout serum antibodies against to pathogens: *Yersinia ruckeri* and *Egtved virus* – Aquaculture 49: 197-208.

Dunier M., Siwicki A.K. 1994a – Effects of environmental contaminations and chemotherapeutics on fish defense mechanisms – Arch. Pol. Fish. 2: 21-54.

Dunier M., Siwicki A.K. 1994b – Effects of lindane exposure on rainbow trout *Oncorhynchus mykiss* immunity. I. Effect of lindane on antibody-secreting cells (ASC) measured by ELISPOT assay – Ecotoxicol. Environ. Safety 27: 1-6.

Ellis A.E. 1988 – Current aspects of fish vaccination – Dis. Aquat. Org. 4: 159-163.

Jeney Z., Jeney G., Olah J., Siwicki A., Danko I. 1986 – Propanidid, a new anaesthetic for use in fish propagation – Aquaculture 54: 149-156.

Kazuń K., Siwicki A.K. 2001 – Propiscin – a safe new anaesthetic for fish – Arch. Pol. Fish. 9: 183-190.

Nikl L., Albright L.J., Evelyn T.P.T. 1991 – Influence of seven immunostimulants on the immune response of coho salmon to *Aeromonas salmonicida* – Dis. Aquat. Anim. 12: 7-12.

Siwicki A.K., Dunier M. 1993 – Quantification of antibody secreting cells to *Yersinia ruckeri* by ELISPOT assay after *in vivo* and *in vitro* immunization of rainbow trout (*Oncorhynchus mykiss*) – Vet. Immunol. Immunopathol. 37: 73-80.

Siwicki A.K., Klein P., Morand M., Kiczka W., Studnicka M. 1998a – Immunostimulatory effects of dimerized lysozyme (KLP-602) on the nonspecific defence mechanisms and protection against furunculosis in salmonids – Vet. Immunol. Immunopathol. 61: 369-378.

Siwicki A.K., Morand M., Fuller J.C., Nissen S., Kazuń K., Głąbski E. 2001 – Influence of HMB (beta-hydroxy-beta-methylbutyrate) on antibody secreting cells (ASC) after in vitro and in vivo immunization with the anti-*Yersinia ruckeri* vaccine of rainbow trout (*Oncorhynchus mykiss*) – Vet. Res. 32: 491-498.

Siwicki A.K., Morand M., Klein P., Studnicka M., Terech-Majewska E. 1998b – Modulation of nonspecific defence mechanisms and protection against diseases in fish – Acta Vet. Brno 67: 323-328.

Smitt G.L., Hatting J., Burger A.P. 1979 – Haematological assessment of the effects of the anaesthetic MS-222 in natural and neutralized form in three freshwater fish species: intraspecies differences – J. Fish Biol. 15: 645- 653.

Studnicka M., Siwicki A.K., Morand M., Rymuszka A., Bownik A., Terech-Majewska E. 2000 – Modulation of nonspecific defence mechanisms and specific immune responses after suppression induced by xenobiotics – J. Appl. Ichthyol. 16: 1-7.

Trzebiatowski R., Stepanowska K., Siwicki A.K., Kazuń K. 1996 – The study of an usefulness of the confection Propiscin to anaesthesia of European wells (*Silurus glanis*) – Komun. Ryb. 1: 14-18 (in Polish).

Veenstra R.S., Balon E.K., Flegler-Balon C. 1987 – Propanidid, a useful anaesthetic for studying blood circulation in early development of fish – J. Can. Zool. 65: 1286-1289.

STRESZCZENIE

ZASTOSOWANIE ŚRODKÓW ANTYSTRESOWYCH W AKWAKULTURZE: WPŁYW PROPISCINU NA EFEKTYWNOŚĆ SZCZEPIONKI PRZECIW *YERSINIA RUCKERI* U PSTRĄGA TĘCZOWEGO *ONCORHYNCHUS MYKISS* (Wal.)

Stosowanie swoistych szczepionek jest najbardziej efektywną metodą ochrony zdrowia ryb. Jednakże przy użyciu aktualnych technik immunizacyjnych uzyskiwane efekty szczepień nie są w pełni zadowalające. Prowadzone są badania nad zwiększeniem efektywności szczepień przez stosowanie środków antystresowych, eliminujących negatywny wpływ stresu polietiologicznego na organizm ryb. Celem prezentowanych badań było określenie wpływu znieczulenia ogólnego preparatem Propiscin (IRS) na efektywność szczepionki przeciwko *Yersinia ruckeri* podawanej w iniekcji lub immersji pstrągom tęczowym (*Oncorhynchus mykiss*). Wyniki porównywano z uzyskanymi bez zastosowania znieczulenia ogólnego. W celu sprawdzenia efektywności szczepienia 21 dnia po wakcynacji ryby poddano zakażeniu żywymi bakteriami *Y. ruckeri*. Bezpośrednio przed i 21 dni po zakażeniu od części ryb pobierano krew i nerkę głowową. Określano ogólną ilość komórek produkujących przeciwciała (ISC), poziom komórek produkujących swoiste przeciwciała (ASC), poziom immunoglobulin (Ig) w surowicy oraz miano swoistych przeciwciał anti-*Y. ruckeri*. Po zakażeniu eksperymentalnym kontrolowano stan kondycyjny oraz rejestrowano liczbę śnięć. Uzyskane wyniki przedstawiono na odpowiednich wykresach (rys. 1, 2, 3, 4, 5).

Wyniki badań jednoznacznie wykazały, że u ryb poddanych szczepieniu zarówno w iniekcji jak i w immersji w pełnym znieczuleniu ogólnym stwierdzono statystycznie istotnie wyższą odpowiedź immunologiczną określaną poziomem ISC, ASC, Ig oraz mianem swoistych przeciwciał. Również obserwowano znacznie niższy procent śmiertelności po zakażeniu eksperymentalnym u ryb poddanych immunizacji w znieczuleniu ogólnym.

CORRESPONDING AUTHOR:

Prof. dr hab. Andrzej K. Siwicki
Instytut Rybactwa Śródlądowego
Zakład Patologii i Immunologii Ryb
Żabieniec
ul. Główna 48
05-500 Piaseczno
Tel./Fax: +48 22 7562490; e-mail:aksiw@infish.com.pl