Arch. Ryb. Pol.	Archives of Polish Fisheries	Vol. 8	Fasc. 1	5-14	2000

THE INFLUENCE OF ZINC ON SPERM MOTILITY OF COMMON CARP - A COMPUTER ASSISTED STUDIES

Jarosław Chyb, David E. Kime, Tomasz Mikołajczyk, Paweł Szczerbik, Piotr Epler*

Department of Ichthyobiology and Fisheries, Agricultural Academy, Kraków,
*The University of Sheffield, Sheffield SIO 2UQ, United Kingdom

ABSTRACT. The influence of zinc on common carp sperm motility was investigated using a computer assisted sperm analysis (CASA) as well as subjective microscopic measurements of time of motility. The analysis by CASA method consisted of 3 parameters - curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL), The following zinc concentrations were used: 10, 50, 100, 200, 500, 1000 and 2000 ppm. Both, CASA and subjective microscopic observations of sperm motility showed that zinc decreased the motility of sperm, having lethal effects at concentrations of 500 ppm (as analysed by computer assisted methods) or 200 ppm (as evaluated using microscopic observations of time of motility). Moreover, the computer-assisted analysis demonstrated that zinc had the strongest inhibitory influence on VSL, suggesting the possibility of its negative impact on the fertilisation rate. CASA method seems to be more reliable than classical microscopic observation of sperm activity.

Key words: CARP, SPERM, CASA, ZINC, TOXICITY

INTRODUCTION

The increase of the industrialisation and intensive agriculture is one of the results of the economical growth in developed countries. This, in turn, is responsible for the robust growth of air, water and soil pollution. Since the inland water and seawater are the final target of accumulation of pollutants from industry and agriculture, it is supposed that in water the concentration of pollutants will be relatively high. This in turn can negatively affect the fish, which are one of the food resources for human beings and animals. The result of fish exposition to extremely high contaminant concentration is easy to predict, however it is difficult to evaluate the effects of sublethal pollutant doses on different aspects of fish biology, including physiology of fish. This «hidden» impact of low pollutant concentration, affecting many tissues and organs in fish, requires development of fast and objective method of evaluation of the negative influence of water contaminants on fish organisms.

Among different pollutants, heavy metals are of important interest. According to Forster and Muller (1974) heavy metals are the elements whose density is higher than 6 g cm^{-3}. One of such elements is zinc. Zinc is abundant in the inland waters. Polish

rivers transport each year to the Baltic Sea around 1200 tons of this metal (Protasowicki 1991). In south-west Poland the zinc concentration in rivers reaches up to 530 μg dm^{-3}, and the average zinc concentration is 98 μg dm^{-3}, while in fish ponds the concentration of this metal reaches l mg dm^{-3} (Szulkowska-Wojaczek et al. 1992). Such a high concentration, at least in part, is the result of agricultural fertilisation. Mineral and organic fertilisers contain relatively high doses of zinc compounds. For example, phosphoric fertilisers contain 67.2-206.9 ppm of zinc in dry mass, while in the cow dung the zinc content reaches 250 ppm (Szulkowska-Wojaczek et al. 1992).

Zinc is a necessary microelement in numerous physiological and biochemical processes. In fish tissues it is an element of many enzymes or it makes the complexes with enzymes, increasing their activity. Zinc takes part in the processes associated with tissue growth, cellular resistance, osteogenesis (Cousins 1985) and is necessary in many reproductive processes (Mills 1988).

Zinc possesses strong accumulative properties, however its content varies among different fish tissues and organs (Szulkowska-Wojaczek et al. 1992). The highest zinc concentration in carp was observed in bones and scales, while in the liver and kidney the zinc content is several times lower. On the other hand muscle tissue has a relatively low concentration of this metal, comparing to the above tissues and organs. Moreover, also fish species differ in the zinc content. Common carp has about 15 times higher concentration of this metal in several tissues than many other fish species, like tilapia, grass carp or silver carp (Jeng and Sun 1981).

Zinc has a negative influence on numerous physiological functions in fish, including reproduction (for review see Kime 1998). Zinc used in the concentrations which didn't affect growth, survival ratę or maturation has drastically decreased fertility in *Pimephales promelas Rąfinesque* (Brungs 1969). Speranca et al. (1977) have found that 9-day exposure of *Brachydanio rerio* to water contaminated with zinc compounds has led to the delay of spawning and to the decrease of egg viability. In *Puntius conchonius* exposed to zinc for 60-120 days Kumar and Pant (1984) observed the inhibition of the spermatogenesis and the atresion of ovaries. Munkittrick and Dixon (1989) noticed the decrease of diameter of laid eggs and subsequent deformations of larvae.

Also the investigations performed in our laboratory on *Poecilia reticulata* demonstrated that the presence of zinc compounds in water as well as in the fish food led to a decrease of sperm volume, decreased fertility and the percentage of hatching (Bieniarz et al. 1994).

Since the quality of fish semen is one of the main factors responsible for fertilisation and subsequent production of health hatch, therefore heavy metals, such as zinc, can negatively act on reproduction processes in direct manner (i. e. during the spawning) or indirectly (on the processes of spermatogenesis). Until mid-90'ies such investigations on the effects of pollutants on the quality of sperm were performed using a subjective microscopic evaluation of time of sperm motility. However, fish sperm, contrary to that of mammals, activates in water for only short period. Therefore it was necessary to find a good, objective and precise method to evaluate different parameters characterising sperm motility of fish. Recently, the computer assisted sperm analysis (CASA), widely used in the medicine, has been successfully involved to analyse the sperm motility in fish (Toth et al. 1995, Ebrahimi et al. 1995, Kime et al. 1996, Rurangwa et al. 1998). Therefore, the aim of our studies was to evaluate the impact of zinc on sperm motility of common carp (*Cyprinus carpio* L.) using the computer assisted sperm analysis (CASA) as well as the subjective microscopic observations of sperm motility.

MATERIAL AND METHODS

ANIMALS

The experiment was conducted in June in the Department of Ichthyobiology and Fisheries, Agricultural University of Kraków, Poland. In the experiment, 7 mature, spermiating male carps were used. Prior to experiment fish were caught from conventional earth ponds, then transferred to concrete flow-through basins. The fish were acclimated to 20°C under the natural photoperiod for at least 3 days.

SPERM SAMPLING

Anaesthetized fish were wiped using a wet cloth, then stripped of milt using a l-ml sterile syringe. The resulted sperm from each fish was immediately subjected to dilutions with different pollutant concentrations.

EXPERIMENTAL DESIGN

In the experiment the two-step method of sperm dilution was used according to Billard and Cosson (1992). In the first step, the sperm was diluted 100-fold in 10-ml polypropylene tubes in the basal solution (control group) consisting of KCI 200 mM - Tris 30 mM pH=8.0 or in the basal solution containing the following concentrations

of pollutant ($ZnCl_2$ - P.O.Ch., Poland) - 10, 50, 100, 200, 500, 1000 and 2000 ppm (expressed as the concentration of pure metal). After the gentle mixing the tubes were incubated for 2 hours at 4° C. In the second step, just before registration, 1 µl of each mixture was placed on a polyvinyl alcohol (PVA) coated microscope slide (Chance Propper Ltd., UK) and mixed with 20 µl of distilled water to total dilution of 2000.

COMPUTER ASSISTED SPERM ANALYSIS (CASA)

Sperm movement was recorded on a videorecorder (VQ 336, Samsung, South Corea) for 2 mins from the moment of final dilution using a videocamera (TK 1280E, JVC, Japan) attached to an inverted microscope with a phase-contrast objective lens (Wilovert S, Hund, Germany). Videotapes were analysed using a Hobson sperm tracker (Hobson Tracking Systems Ltd., Sheffield, UK), from 20 seconds after the mixing point (to allow for focusing and stabilisation of water solution movement) for 15 seconds. The parameters assessed were:

VCL - curvilinear velocity (µm sec^{-1}), the sum of the incremental distances moved

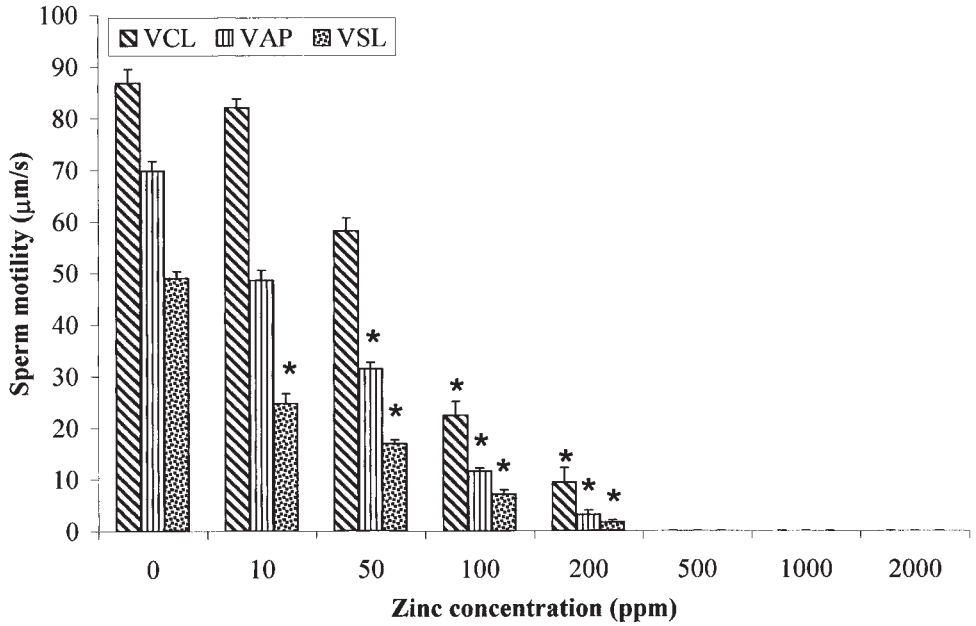

Fig. 1. The effects of different zinc concentrations on curvilinear velocity (VCL), average path velocity (VAP) and straight line velocity (VSL) of common carp sperm, The values are expressed as mean (main bars) ± SEM (error bars). * - statistically different vs. appropriate control group ($p<0.05$)

in each frame along the sampled path divided by the total time of the track; VSL - straight line velocity (μm sec^{-1}), the straight line distance between the start and end points of the track divided by the time of the track; VAP - angular path velocity (μm sec^{-1}), a derived path based on an average number of points and divided by the time of the track. Details of the analysis are given in Kime et al. (1996).

TIME OF SPERM MOTILITY

The time of sperm motility was subjectively measured using a timer, from the moment of sperm activation until the time when about 80% of sperm were immobile.

STATISTICS

The data on VCL, VAP and VSL was stored to disk and was subsequently analysed using the analysis of variance followed by Duncan's multiple range test. The differences between control and experimental groups were considered significant for $P<0.05$. The data on the time of sperm motility were subjected to the same statistical analysis.

RESULTS

CURVILINEAR VELOCITY (VCL)

The carp sperm motility in the control solution had an average VCL motility of 86.84 ± 2.67 $\mu m\ s^{-1}$. The incubation of sperm with zinc at concentrations of 10 and 50 ppm didn't have a significant influence on sperm motility, however at higher concentrations (100 and 200 ppm) this metal significantly affected the VCL parameter of sperm motility. Zinc used at the concentrations of 500, 1000 and 2000 ppm completely inhibited carp sperm activity (Fig. 1).

AVERAGE PATH VELOCITY (VAP)

The potency of zinc ions to inhibit the average path velocity (VAP) was higher than in case of VCL. Even the dose of 50 ppm of zinc significantly inhibited sperm velocity in comparison with control group where the average value was 69.77 ± 1.92 μm s^{-1}. Increasing the zinc concentration led to a subsequent diminution of average path velocity up to zinc concentration of 500 ppm and higher where no motility was found (Fig. 1)

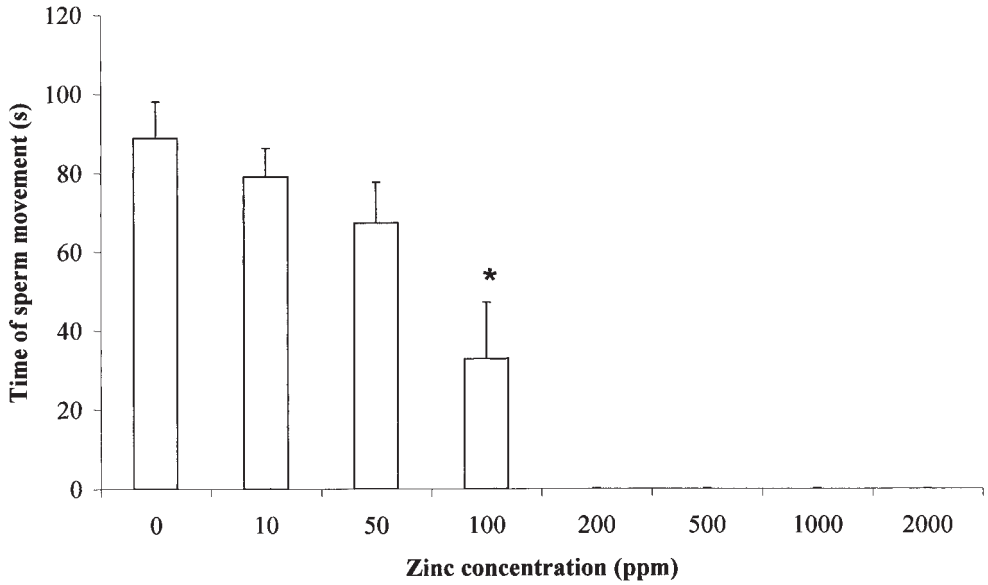

Fig. 2. The average time of sperm motility in different concentrations of zinc. The values are expressed as mean (main bars) ± SEM (error bars). * - statistically different vs. appropriate control group ($p<0.05$)

STRAIGHT LINE VELOCITY (VSL)

In the control group, the straight line velocity (VSL) had an average value of 49.01 ± 1.36 μm s^{-1}. The incubation of carp sperm with all the zinc concentrations used in the experiment led to a significant diminution of this parameter. The 3 highest zinc doses (i. e. 500, 1000 and 2000 ppm) completely inhibited the motile activity of carp sperm (Fig. l)

TIME OF ACTIVITY (AT)

The sperm in control conditions moved for 89 ± 9.21 s, whereas similarly to the results of other motility parameters, in experimental groups the higher concentrations of zinc were used the shorter was the time of sperm viability up to the concentration of 200 ppm and higher, where no sperm motility was observed (Fig. 2)

DISCUSSION

In the latest decades of the twentieth century the attention on the problem of contamination increased significantly. However the research concerning this topic was

focused on the aspects of general impact of pollutants on environment. There is still lack of data on the effects of heavy metals on animal physiology. This, of course, includes the fish, organisms being highly exposed to the toxic influence of water contaminants. Hence, our investigations aimed to evaluate the influence of sublethal doses of zinc on one of the factors responsible for the success of reproduction - sperm motility.

The results of our experiments clearly show that zinc has a negative influence on motility of carp spermatozoa. The analysis of sperm motility using a computer-assisted analysis as well as the subjective evaluation of the time of sperm activity demonstrates that the increase of zinc concentration provokes the diminution of sperm motility.

The analysis of sperm motility using 3 parameters - VCL, VAP and VSL, showed that lethal dose of zinc was 500 ppm. Similar investigations were performed on the African catfish *(Clarias gariepinus*), but the toxic effect of zinc on the sperm of this species was visible at the concentration of 2000 ppm (Ebrahimi et al. 1996). The differences between these results are potentiated by the fact that African catfish sperm was incubated in the presence of zinc for the period of 24 hours. The explanation of these differences can be the possibility of higher zinc uptake in case of carp than in catfish. Jeng and Sun (1981) showed that carp possessed much stronger ability to accumulate zinc compounds than other freshwater species. Therefore it is highly possible that carp sperm has much higher rate of zinc absorption that of African catfish.

Zinc is an element necessary in some reproductive processes (Mills 1988; Motossian 1991), however our studies show that in higher concentrations it can have negative effects on sperm motility. Ebrahimi et al. (1996) showed that zinc ions can eliminate calcium ions from the specific binding sites in sperm. This coincides with the results obtained by Busselberg (1995) demonstrating that calcium channels are the targets of zinc ions. Because the deficiency of calcium blocks the motile activity of spermatozoa (Maisse et al. 1995), it becomes clear that at least one of the effects of zinc on sperm is the displacement of calcium, necessary for activation of fish spermatozoa in water.

Computer assisted sperm analysis (CASA) of carp sperm motility also showed that zinc has unequal influence on the different velocity parameters. This heavy metal was less harmful on curvilinear velocity - it didn't affect the sperm motility at doses of 10 and 50 ppm of zinc. On the other hand this factor evoked a significant decrease of VAP at dose of 50 ppm and VSL even in the concentration of 10 ppm (less than 50% of average control value). This demonstrates that at low doses zinc doesn't significantly

reduce the sperm motility, but it shortens the distance of sperm movement. In other words, carp spermatozoa exposed to zinc pass shorter straight distance than intact sperm.

This fact seems to be very important, since it is suggested that the success of fertilisation depends on the distance from the sperm to ovum (Moore and Akhondi 1996). Thus the decrease of VSL can evoke the subsequent decrease in the fertilisation rate.

Similar results to obtained using computer assisted analysis were found by the evaluation of the time of sperm motility. However, using this method, sperm motility was observed in the zinc concentration of up to 100 ppm. Because the results obtained using CASA are much more coincident, it is possible that our subjective analysis was less reliable. This conclusion is in accordance with the results obtained by Centola (1996), who demonstrated that CASA gives much more detailed results which are less charged by errors than the manual microscopic observation of sperm number and motility.

The above results confirm also the suggestion of Kime (1996) that fish spermatozoa are good bioindicators of water pollution. Therefore, taking into consideration short time of exposition and motile activity after the contact with water, fish spermatozoa can be a good model for the investigations on the toxic influence of water contaminants.

REFERENCES

Alabaster J. S., Lioyd R. 1980 - Water quality criteria for freshwater fish - FAO, Butterworths, London-Boston, 3-295

Bieniarz K., Epler P., Sokołowska-Mikołajczyk M. 1994 - Effect of zinc on guppy *(Poecilia reticulata)* reproduction - Pol. Arch. Hydrobiol. 41 (4): 489-493

Brungs W. A. 1969 - Chronic toxicity of zinc to the fathead minnow *(Pimephales promelas* Rafinesque) - Trans. Am. Fish. Soc. 98: 272-279

Busselberg D. 1995 - Calcium channels as target sites of heavy metals - Toxicol. Lett. 82/83:255-261

Centola G. M. 1996 - Comparison of manual microscopic and computer- assisted methods for analysis of sperm count and motility - Arch. of Androl. 36: 1-7

Cousins R. J. 1985 - Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin - Amer. Physiol. Soc. 65,2: 238-309

Ebrahimi M., Nysten K., Roelants I. Ollevier F., Kime D. E. 1996 - Use of computer assisted sperm anałysis (CASA) for monitoring sperm quality; application for determining effects of heavy metal pollutants: 47-49 - In: Larvi 95 - Fish & Shellfish Larviculture Symposium, European Aquaculture Soc., Special Publication No. 24 (Ed.) P. Lavens, E. Jaspers and I. Roelants, Gent, Belgium

Forstner U., Muller G. 1974 - Schwermetalle in Flusses und Seen als Ausdruck der Umweltverschmutzung - Springer-Verlag Berlin, 225

Jeng S. S., Sun L. T. 1981 - Effects of dietary zinc levels on zinc concentrations in tissue of common carp - J. Nutr. 134-140

Kabata-Pendias A., Pendias H. 1979 - Pierwiastki śladowe w środowisku biologicznym - Wyd. Geologiczne Warszawa, 300

Kime D. E. 1998 - Endocrine disruption in fish - Kluwer Academic Publishers, USA

Kime D. E. Ebrahimi M., Nysten K., Roelants I., Moore H. D. M., Ollevier F. 1996 - Use of computer assisted sperm analysis (CASA) for monitoring sperm quality; application for determining effects of heavy metals - Aqua. Toxicol. 36: 223-237

Kumar S., Pant S. C. 1984 - Comparative effects of the sublethal poisoning of zinc, copper and lead on the gonads of the teleost *Puntius conchonius* Ham - Toxicol. Lett. 23: 189-194

Lloyd R. 1960 - The toxicity of zinc sulphate to rainbow trout - Ann. Appl. Biol. 48: 84-94

Maisse G., Billard R., Andre F., Cosson M., Le Gac F. 1995 - Amelioration de la motilite des spermatozoides testiculaires de neomales de truite commune *(Salmo trutta)* au debut de la periode de reproduction - Aquat Living Resour. 8: 191-194

Millar M., Elcoate P., Fischer M., Mawson C. 1960 - Effect of testosterone and gonadotrophin injections on the sex organ development of zinc deficient male rats - Can. J. Biochem. Physiol. 38: 1475-1477.

Mills C. S. 1988 - Zinc in human biology - Springer, Berlin. 307 pp

Matossian M. K. 1991 - Fertility decline in Europe, 1875-1913 was zinc deficiency the cause? - Persp. Biol. Med. 34: 604-616

Moore H. D. M., Akhondi M. A. 1996 - Fertilising capacity of rat spermatozoa is correlated with decline in straight-line velocity measured by continuous computer-aided sperm analysis: epididymal rat spermatozoa from proximal cauda have a greater fertilising capacity *in vitro* than those from the distal cauda or vas deferens - J. Androl. 17: 50-60

Munkittrick K. R., Dixon D. G. 1989 - Effects of natural exposure to copper and zinc on egg size and larval copper tolerance in white sucker *(Castostomus commersoni)* - Ecotox. Environ. Safety 18: 15-26

Pasternak K. 1973 - Występowanie i zmienność mikroelementów w wodzie w podłużnym przekroju rzeki Nidy - Acta Hydrobiol. 15: 145-166, 357-378

Protasowicki M. 1991 - Long-term studies on heavy metals in aquatic organisms from the river Odra mouth area - Acta Ichthyol. et Piscatoria. XXI

Rurangwa E., Roelants I., Huyskens G., Ebrahimi M., Kime D.E., Ollevier F. - 1998 – The minimum effective spermatozoa to egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilisation ability in the African catfish (*Clarias gariepinus*, Burchell 1822) - J. Fish. Biol. 53: 402-413

Speranca A. W., Seeley V. A., Perlmutter A. 1977 - The effect of sublethal concentrations of zinc on reproduction in the zebrafish, *Brachydanio rerio* Hamilton-Buchanan - Environ. Poll. 12: 217-222

Szulkowska-Wojaczek E., Marek J., Dobicki W., Polechoński R. 1992 - Metale ciężkie w środowisku stawowym - Zesz. Nauk. AR Wrocław, Zootechnika XXXVII, 218: 7-25

Toth G. P., Christ S. A., McCarthy H. W., Torsella J. A., Smith M. K. 1995 - Computer-assisted motion analysis of sperm from the common carp - J. Fish Biol. 47: 986-1003

STRESZCZENIE

WPŁYW CYNKU NA RUCHLIWOŚĆ SPERMY KARPIA - BADANIA Z WYKORZYSTANIEM ANALIZY KOMPUTEROWEJ

Niniejsza praca miała na celu zbadanie wpływu cynku na ruchliwość plemników karpia. Dokonano pomiaru ruchliwości za pomocą wspomaganej komputerowo analizy plemników (CASA) przy wykorzystaniu 3 parametrów charakteryzujących poruszanie się plemników -VCL, VAP i VSL. Przeprowadzono również pomiar czasu ruchliwości plemników metodą obserwacji mikroskopowych. W doświadczeniach zastosowano następujące stężenia cynku:

10, 50 100, 200, 500, 1000 oraz 2000 ppm. Zarówno komputerowa analiza ruchliwości jak i obserwacje mikroskopowe wykazały, że cynk hamująco wpływa na ruchliwość plemników wykazując efekt letalny

przy koncentracji 500 ppm (w przypadku metody komputerowej CASA) lub 200 ppm (przy obserwacjach mikroskopowych). Wykazano ponadto, że cynk w największym stopniu hamuje ruchliwość plemników mierzoną parametrem VSL, co sugeruje prawdopodobny negatywny wpływ cynku na zdolność plemników do zapłodnienia. Komputerowa analiza ruchliwości plemników jako bardziej powtarzalna i obarczona o wiele mniejszym błędem pomiaru wydaje się być lepszą metodą niż obserwacja mikroskopowa.

ADRESY AUTORÓW:

Prof. Dr hab. Piotr Epler
Dr hab. Tomasz Mikołajczyk
Dr Jarosław Chyb
Mgr Paweł Szczerbik
Katedra Ichtiobiologii i Rybactwa
Akademia Rolnicza w Krakowie
ul. Prof. Spiczakowa 6
30-149 Kraków

Dr David E. Kime
Department of Animal and Plant Sciences
The University of Sheffield
Sheffield S102UQ
Wielka Brytania