Ammonia-induced cellular and immunological changes in juvenile *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis*

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SUMMARY

Immunological and structural changes in the thymus and pronephros of *Cyprinus carpio* infected with the blood fluke, *Sanguinicola inermis* for 30 days, and exposed to 0·5 mg NH_4^+/l for 48 and 168 h were investigated. Ultrastructural observations revealed cell disruption and highly vacuolated cytoplasm in the thymus. Of the cells that remained intact there was a significant increase in thrombocytes after 48 h exposure to the pollutant. In addition, there was a decrease in lymphocytes following exposure to ammonia at both time-periods studied. In contrast the pronephros of fish exposed to the pollutant underwent relatively mild changes in cellular architecture although ammonia and time of exposure had significant effects on the proportions of several leucocyte types. A significant decrease in neutrophils, thrombocytes and lymphocytes occurred in fish exposed to the pollutant for 168 h. Pronephric lymphocyte stimulation (cpm) by Con A and PWM increased *in vitro*, whereas the stimulation index was reduced in infected fish exposed to ammonia. Changes in the immune organs of *S. inermis*-infected carp treated with pollutant were both organ- and time-specific. The possible reasons for this are discussed and significance in relationship to parasitization assessed.

Key words: *Cyprinus carpio*, *Sanguincola inermis*, ammonia, pathology.

INTRODUCTION

Although in the natural environment, organisms are often subjected to a variety of stressors simultaneously, for example infections and pollutants, the effects on the hosts have not been ascertained (Khan & Thulin, 1991; Wester, Vethaak & Van Muiswinkel, 1994; Hoole, 1997).

Ammonia, a pollutant which is present at sublethal levels in many freshwater systems (Alabaster & Lloyd, 1982), occurs in both the unionized toxic form (NH_3) and the less toxic ionized form (NH_4^+) and is produced as a metabolite from the natural degradation of nitrogenous organic material. The toxicity varies with pH, temperature and dissolved oxygen (Lloyd, 1961, 1992; Trussell, 1972) and is known to cause pathological lesions in teleosts (Thurston *et al*. 1984; Banerjee & Bhattacharya, 1997). Ammonia also affects the permeability of the cell membrane to ions in the blood plasma which, in turn induces net movements of ions and water into the cells leading to their swelling and rupture. Vasoactive amines, released through cell rupture, increase the blood flow, which in turn facilitates the redistribution of leucocytes (Dhabhar *et al*. 1995). Although the toxic effects of ammonia on fish have been studied extensively (Lloyd, 1961; Lloyd & Herbert, 1960; Lloyd & Orr, 1969; Banerjee & Bhattacharya, 1997; Magaud *et al*. 1997; Linton, Reid & Wood, 1997) the effect of metazoan parasites on the immune response in fish has received little attention. In addition, the joint effects of pollutants and parasitism on the immune system of the host has not been ascertained. Our previous studies have revealed that carp, *Cyprinus carpio*, produces an immune response against the pathogenic blood fluke, *Sanguinicola inermis*. This is manifested in an intense inflammatory response which encapsulates the parasite eggs (Richards *et al*. 1994*a*) and affects various immune parameters e.g. cellular composition of immune organs (Richards *et al*. 1994*b*) and stimulation of lymphocytes (Richards *et al*. 1996). An understanding of the immunological interactions that occur between *C. carpio* and *S. inermis* has enabled us to carry out the first study on the effects of simultaneous infection with this parasite and exposure to ammonia on the immune status of *C. carpio*. The effects on the ultrastructure and cellular composition of two immune organs, the thymus and the pronephros, and on pronephric lymphocyte stimulation are reported.

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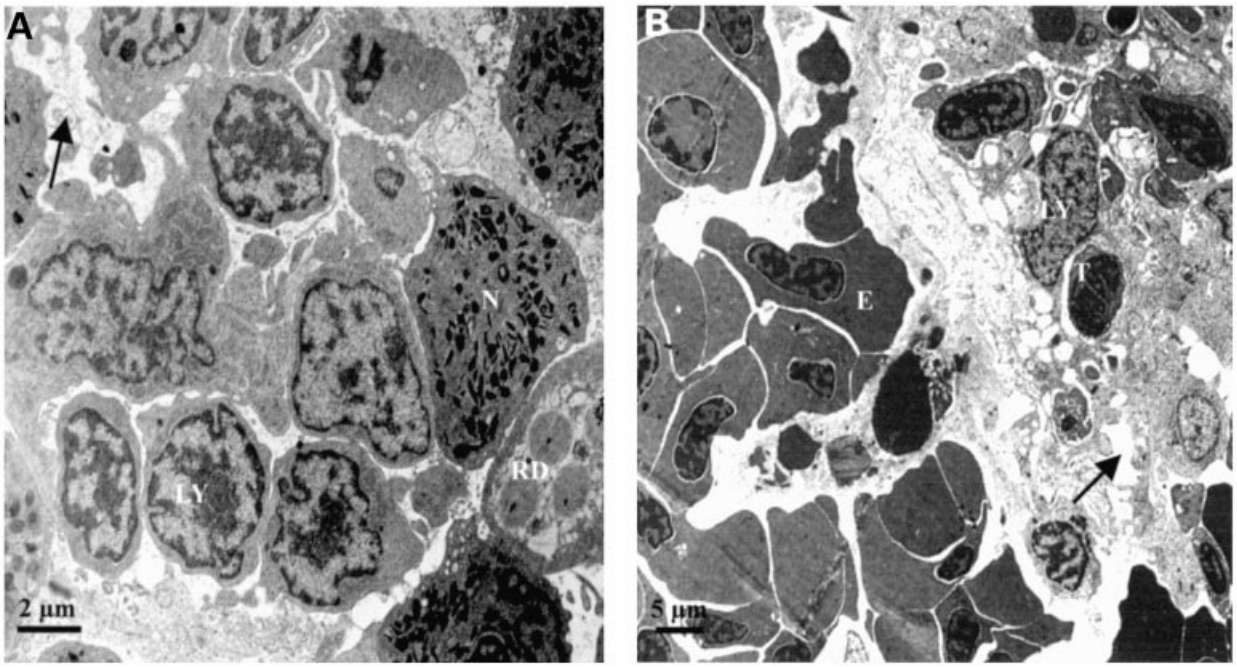


Fig. 1. Transmission electron microscopy of *Sanguinicola inermis*-infected pronephric (A) and thymic (B) tissue of *Cyprinus carpio* kept in unpolluted water. Note intercellular spaces (arrow) in pronephros tissues and vacuolation (arrow) in the thymus (B). E, erythrocytes; LY, lymphocytes; N, neutrophils; RD, rodlet cells; T, thrombocytes.

MATERIALS AND METHODS

Source and maintenance of carp

Twenty-four carp fry (3·5 g ± 0·56) cultured from the same genetic stock, were obtained from Fair Fisheries, Shropshire, UK. The fry were acclimatized for 4 weeks in filtered, aerated and dechlorinated water in polyethylene tanks (225 l) at 20 ± 1 °C and a photoperiod of 12 h light : 12 h dark. Fish were fed a commercial pellet diet (Mazuri TM, Zoo Foods Ltd) twice a week.

Source of infected snails

The intermediate snail host, *Lymnaea peregra*, collected from the margins of Maiden Erlegh Pond, Reading, UK, was maintained in aerated filtered pond water in transparent polyethylene aquaria (10 l) at 20 °C and a photoperiod of 12 h light : 12 h dark. Snails were fed on lettuce leaves supplemented with calcium and screened for infections of *S. inermis* twice a week. Individual snails were transferred into glass vials with 20 ml of pond water and the release of cercariae monitored under a dissecting microscope at 19 : 00 h and 20 : 00 h (see Richards *et al*. 1994*a*).

Laboratory infection of carp

Individual fish were exposed to 500 cercariae in 300 ml circular glass vials for 6 h and then transferred in batches of 6 to aerated glass aquaria (30 l), where they were kept for 30 days post-infection (Kirk & Lewis, 1992).

Water quality, exposure system, and maintenance of carp in ammonia

Prior to experimentation, the water quality was analysed twice daily over a period of 4 days to determine the chemical and physical parameters, which may affect the toxicity of ammonia. Replicate samples were taken and acidified with 1 % concentrated HNO_3 (BDH Ltd) and analysed on an Inductive Coupled Plasma Optical Emission Spectrophotometer (ICPOES) and Gas Chromatograph-Mass Spectrophotometer (GC-MS).

Twenty-four infected carp (6 per treatment), infected for 30 days were exposed for 48 or 168 h at 20 °C to aerated, charcoal filtered water (pH 7·6 ± 1) either unpolluted or containing 0·5 ± 0·05 mg NH_4^+/l in the form of anhydrous NH_4Cl (Sigma). Fish were starved 2 days prior and during the exposure period. The pollutant dosing system comprised sealed concrete tanks (approx. 250 l capacity) and a gravity fed water flow-through system (flow rate : 12 ml/sec and a turnover time of 6 h 21 min). The ammonia concentration in the outflow was monitored and additional tanks served to dilute and filter this water to environmentally acceptable concentrations (WHO, UK guidelines in Clark, 1993) before disposal. Pollutant distribution within the tanks was tested with Cresol Red dye prior to experimentation. A constant concentration of pollutant was maintained in the exposure tank using a peristaltic pump (Watson Marlow), applying an NH_4^+ solution (12·5 g NH_4^+/l) at a rate of 0·175 ml/sec. The pH and NH_4^+concentration were monitored (Waterman International Ltd, pH 6–8·1; Aquaquant 14400,

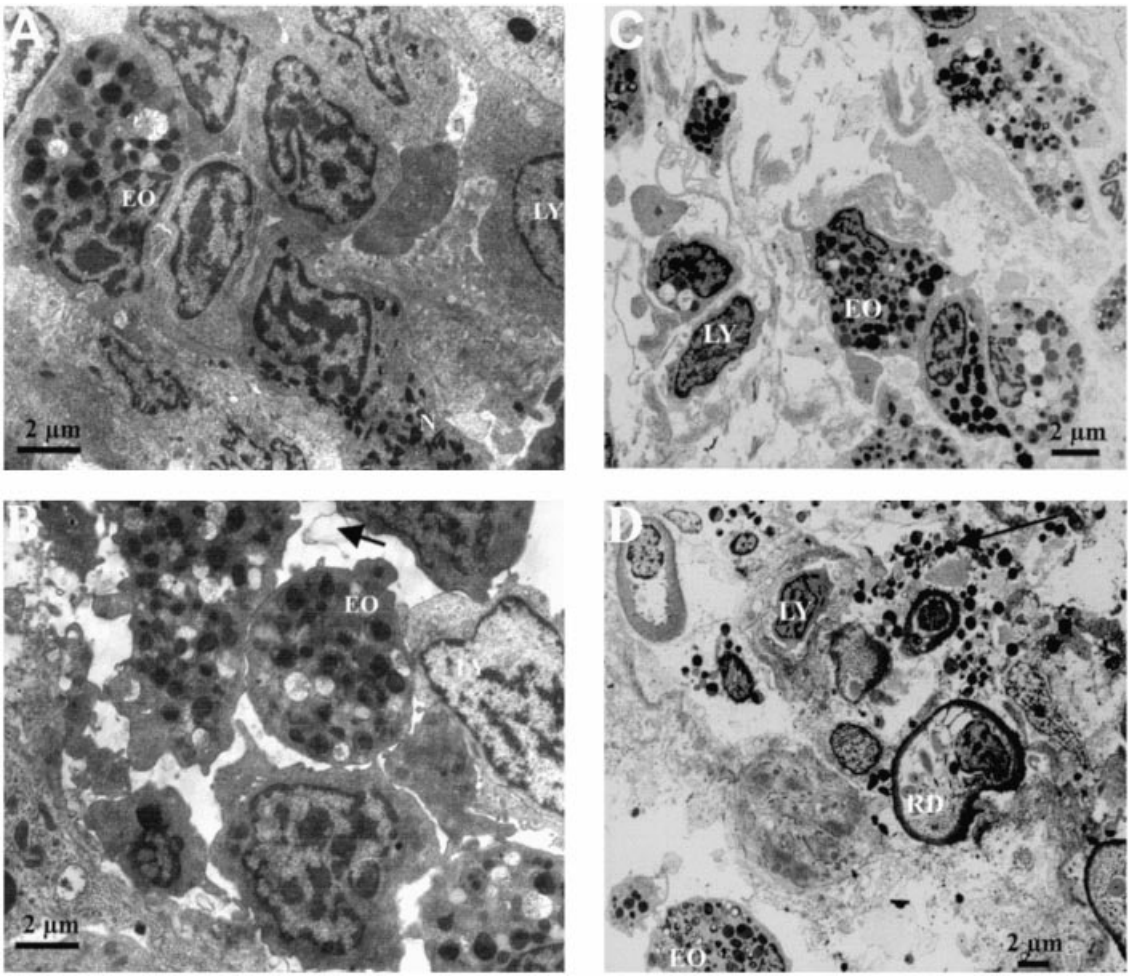


Fig. 2. Transmission electron microscopy of the pronephros (A) and (B) and thymus (C) and (D) in *Sanguinicola inermis*-infected *Cyprinus carpio* fixed after 48 h (A) and (C) and 168 h (B) and (D) exposure to 0·5 mg NH_4^+/l. Note increase in intercellular spaces in the pronephros, some containing concentric whorls (arrow, (B) and disrupted eosinophils (arrow) in thymus after 168 h exposure to ammonia. EO, eosinophils; LY, lymphocytes; N, neutrophils; RD, rodlet cells.

0·05–0·8 mg NH_4^+/l, Merck Reagent Kit) and the system was maintained at constant temperature of 20 °C. After 48 and 168 h exposure to ammonia the pronephros and thymus of individual fish were removed under sterile conditions.

Preparations for TEM

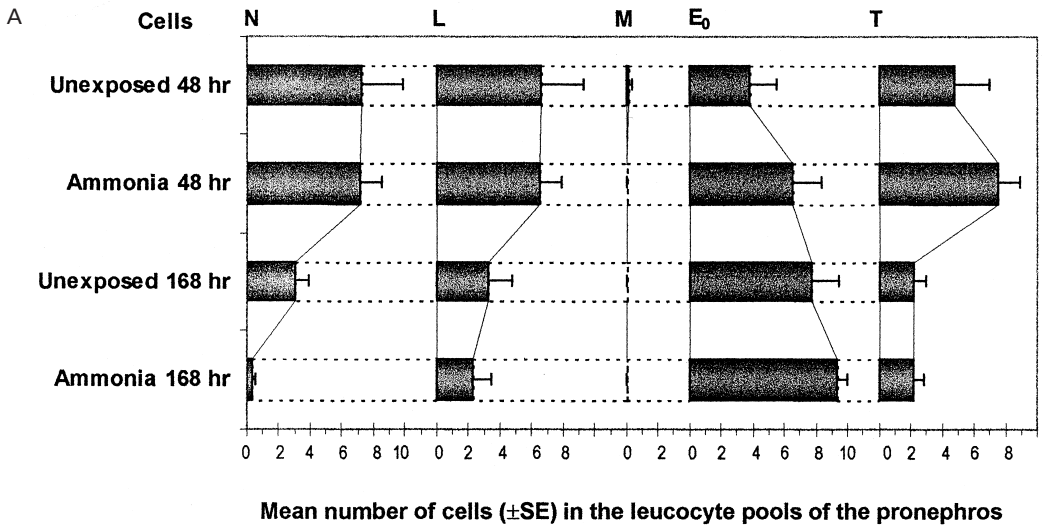
Thymic and pronephric tissues were fixed in 3 % glutaraldehyde in 0·1 M Sorenson's buffer (pH 7·2), washed in the same buffer, post-fixed in 1 % osmium tetroxide, dehydrated in a gradient series of ethanol (30, 50, 70, 90, 100, 100 %) and embedded in Spurrs' resin. Sections were then stained in uranyl acetate and Reynold's lead citrate and viewed under a Hitachi H-600 TEM.

Differential cell counts

Leucocytes and erythrocytes were identified using ultrastructural descriptions given by Cenini (1984) and Richards *et al*. (1994*a*). The number of cells within 3 randomly selected areas (550 μm^2) in 5 ultrathin sections taken from the pronephros and thymus of each carp was counted.

Proliferation assays

The mitogens and pronephric cell suspensions were prepared as previously described by Richards *et al*. (1996). Briefly, leucocyte suspensions (2×10^6 cells/ml) obtained from the pronephros of individual fish were exposed to 0·5 μg/ml ConA or PWM in sterile Leibovitz L-15 culture medium supplemented with 5 % heat-inactivated (20 min at 60 °C) foetal calf serum, penicillin (100 i.u./ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Blastogenic responses were monitored using 0·5 μCi [C6-^{3}H] thymidine incorporation at 20 °C during 72 h. Cells were harvested on a Maxi Cell Harvester (2020C, Wesbart, Prior Laboratory Supplies Ltd) by water lysis onto glass fibre, and radioactivity monitored



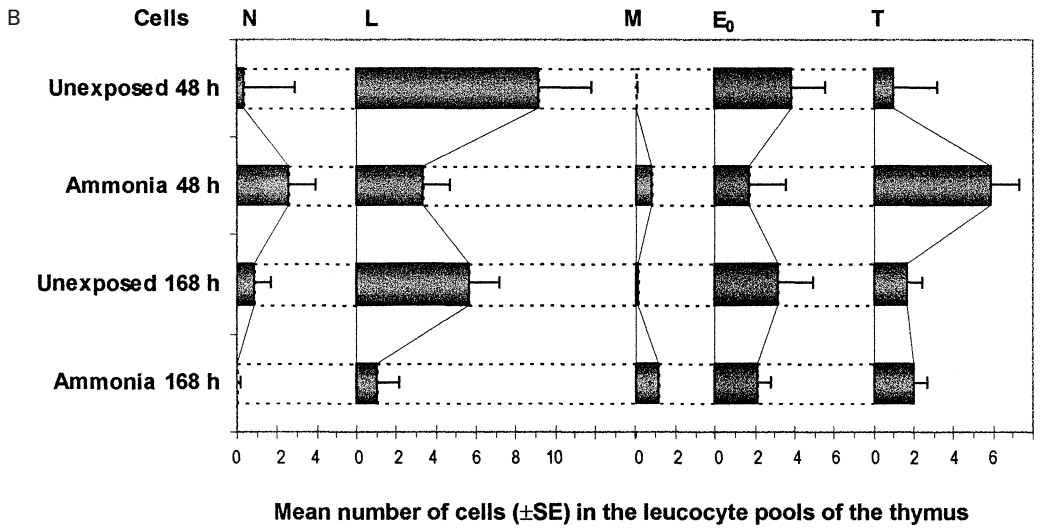


Fig. 3. Differential leucocyte counts in the pronephros (A) and thymus (B) of *Sanguinicola inermis*-infected unexposed and 0·5 mg NH_4^+/l exposed *Cyprinus carpio* after 48 and 168 h. Cells were counted within 3 randomly chosen areas (550 μm^2) in 5 pronephric and thymic tissue sections of individual carp under TEM. N, neutrophils; L, lymphocytes; M, macrophages; E_0, eosinophils; T, thrombocytes.

on a Beta Scintillation Analyser (Tricarb 2200CA, Packard A, Canberra Company) with 4 ml of Ecoscint (National Diagnostics). The index of stimulation or suppression (SI) was calculated as described by Richards *et al.* (1996) using cells which had not been exposed to mitogen as a control.

Statistical analysis

Data on the pronephric and thymic leucocyte numbers were tested by ANOVA using the General Linear Model (GLM) and further analysed using Student's *t*-test and Mann Whitney U depending upon normality. The pronephric lymphocyte proliferation of ammonia-exposed infected and unexposed infected carp, expressed as counts per min, was first analysed for normality with Kolmogorov-Smirnov and changes in lymphocyte stimulation were tested further with GLM.

RESULTS

Physical and chemical parameters in the analysed aquarium water prior to the addition of the pollutant were NH_4^+ (10 μg/l), Cl (24·9 mg/l), PO_4 (2·78 mg/l), SO_4 (25·0 mg/l), Na (10·62 mg/l), K (2·84 mg/l), Mg (19·24 mg/l), Si (4·82 mg/l), Cu (40 μg/l), Zn (10 μg/l), Cd and Al were not detected. The concentration of those parameters that are known to affect NH_3 toxicity were Ca (5·98 mg/l), temperature (20 $\pm$ 1 °C), dissolved oxygen (8·60 mg/l) and pH (6·90 $\pm$ 0·50). The percentage of unionised NH_3 in

Table 1. Con A and PWM stimulation of pronephric lymphocytes from *Sanguinicola inermis*-infected carp exposed to ammonia or unpolluted water for 48 and 168 h (SI = Stimulation Index)

Treatment	cpm Mean ± S.E.	SI Mean ± S.E.
48 h exposure		
Control/Con A	578·44 ± 192·00	1·92 ± 0·49
Ammonia/Con A	1170·10 ± 411·00	1·63 ± 0·78
Control/PWM	763·40 ± 93·90	1·55 ± 0·53
Ammonia/PWM	1255·80 ± 0·80	1·38 ± 0·45
168 h exposure		
Control/Con A	359·80 ± 98·00	1·60 ± 0·45
Ammonia/Con A	538·20 ± 132·00	0·77 ± 0·18
Control/PWM	392·00 ± 204·00	1·15 ± 0·33
Ammonia/PWM	660·00 ± 311·00	1·16 ± 0·78

the aqueous ammonia solution at pH 6·9 and 20 °C was 13·68 % and the calculated concentration of this parameter was 0·0016 mg/l (Trussell, 1972).

Ultrastructural examinations

Thymus and pronephros of infected carp after 48 and 168 h exposure to unpolluted water. In both lymphoid organs of unexposed infected carp a variety of leucocytes was observed (Fig. 1A and B). These included lymphocytes, neutrophils, eosinophils, rodlet cells, macrophages, as well as blast cells and monocytes. Intercellular spaces were also present in both organs (arrow; Fig. 1A) and increasing vacuolation occurred with time in the thymus (arrow; Fig. 1B).

Pronephros of infected carp exposed to 0·5 mg/l NH_4^+ *for 48 and 168 h*. In infected carp that had been exposed to ammonia pollution for 48 h the cytoplasm of pronephric leucocytes was electron dense and granular in appearance. Eosinophils also contained electron-lucent bodies (Fig. 2A). After 168 h exposure to the pollutant large areas of intercellular spaces containing the occasional concentric membrane whorls occurred between leucocytes (arrow; Fig. 2B). Vacuolation also increased in eosinophils (Fig. 2B).

Thymus of infected carp exposed to 0·5 mg/l NH_4^+ *for 48 and 168 h*. The tissue architecture was greatly altered and dissociated after 48 h exposure to ammonia (Fig. 2C). After 168 h of exposure large areas with clusters of eosinophilic granules were observed scattered in the highly vacuolated electron-lucent matrix. These granules appeared to predominate adjacent to rodlet cells, which remained intact (Fig. 2D). Remaining intact eosinophils contained abundant intracellular vacuoles.

Differential cell counts in the thymus and pronephros of infected carp exposed to unpolluted water and water containing ammonia. Significant changes in cell numbers were found, when comparing individual thymic leucocyte pools (Fig. 3B), and cell types of unexposed compared with exposed infected carp ($P < 0·001$). In the pronephros (Fig. 3A) significant differences with cell type ($P < 0·002$), time ($P < 0·001$) and their interaction ($P < 0·001$) were noted. Further statistical analysis of these data revealed that there was no significant difference in the number of neutrophils, thrombocytes and lymphocytes in infected carp exposed to unpolluted water. In contrast, in the pronephros these 3 cell types showed a significant decrease ($P < 0·05$) in infected fish exposed to ammonia for 168 h compared to those exposed for only 48 h. Statistical analysis of cells remaining intact within the thymus (Fig. 3B) revealed that only the thrombocytes showed a significant change in infected fish exposed to the pollutant. Thrombocytes increased after 48 h exposure although they had returned to control levels after 168 h exposure. In addition at both time-periods studied there was the same trend for lymphocyte numbers to be reduced in infected fish exposed to the pollutant.

In vitro-*proliferation of lymphocytes*

The mitogen-induced proliferation (cpm) and stimulation) (SI > 1) of lymphocytes *in vitro* were reduced with all treatments after 168 h exposure of ammonia. In exposed infected fish the lymphocyte proliferation (cpm) was significantly higher after 48 h ($P = 0·023$; Table 1) compared with unexposed infected fish, whereas stimulation (SI) of the cells was reduced in the presence of ammonia. Furthermore, a higher stimulation was found with the mitogen Con A compared with PWM after 48 h. After 168 h exposure the lymphocyte proliferation was suppressed when stimulated with Con A (SI = 0·77 ± 0·18) but unchanged under the same conditions with the mitogen PWM.

DISCUSSION

The ammonia concentration (0·5 mg NH_4^+/l; 0·0016 mg NH_3/l) used during this study was an order of magnitude below the concentration criteria for ammonia as stated for the European Inland Fisheries (0·025 g NH_3/l, Thurston *et al*. 1984; Alabaster & Lloyd, 1982). Although mild lesions occurred in the pronephros, cell disruption, electron-lucent, vacuolated and dissociated cytoplasm and tissue, were observed in the thymus of exposed *S. inermis*-infected carp. The deleterious effects of ammonia on the thymus were further demonstrated with a decrease in the number of lymphocytes and an increase in the blood clotting thrombocytes in this organ. Rodlet cells, which remained intact on exposure to ammonia, were often found surrounded by numerous eosinophilic granules. Rodlet cells

were recently thought to be host-derived cells rather than parasite-derived and are likely to be involved in defence mechanisms (Dezfuli, Capuano & Manera, 1998). Macrophages constitute an important cell population for both specific (antigen processing and presentation) and non-specific (phagocytosis) defence mechanisms and Zelikoff *et al*. (1991) proposed that they may serve as useful bioindicators of pollution. In our studies, however, only a small increase in the number of macrophages in infected fish exposed to ammonia was noted. The apparent differential effects of ammonia on the morphology and cellular composition of the thymus and pronephros of *S. inermis* infected carp may reflect structure and location of both organs. The thymus may be in closer contact with waterborne pollutants being situated close to the opucular cavity. In contrast, relatively minor organ-specific changes were observed in both lymphoid organs of carp exposed to unpolluted water and infected with *S. inermis* infection. This may reflect the fact that the cercariae of *S. inermis* penetrate the carp over its body surface whilst ammonia may primarily affect the gills located in the opercular cavity.

Pronephric lymphocyte stimulation by Con A and PWM in *S. inermis*-infected carp exposed to unpolluted or ammonia-polluted water was lower compared with studies carried out by Richards *et al*. (1996) on healthy carp fry. Of particular note is the increase in lymphocyte proliferation expressed in cpm after 48 h exposure to ammonia and mitogen treatment, which contrasts with a decrease in SI under the same conditions. This suggests that there is an increase in tritiated thymidine incorporation in pronephric lymphocytes from fish exposed to ammonia when the cells were not exposed to mitogens. Additionally, as previously observed by Richards *et al*. (1996), Con A-stimulated T cells of unexposed infected carp had a higher SI compared with PWM stimulated T/B cells.

The variability in the number of leucocytes, their mitogen-induced proliferation and stimulation index in individual carp is unlikely to be due to the methodology or experimental design used as it occurred in both control and ammonia-exposed fish. Variation in these parameters existed even though the carp were derived from the same genetic stock and were maintained under similar conditions. This emphasizes the importance of observing cellular changes in individual fish, rather than establishing a mean response of a specimen group, when assessing the health status of fish exposed to pollution and parasitic infections (Bennett, 1987; Depledge, 1990, 1994; Depledge *et al*. 1993).

In summary, this study represents the first experimental approach to evaluate the impact of pollution on a fish-macroparasite system. The responses in carp infected with *S. inermis* differed when they were exposed to ammonia pollution compared to fish exposed to unpolluted water. The organ-specific differences with respect to morphology and cellular composition were noted between the pronephros and thymus. In addition, the pronephric lymphocytes had increased mitogen-induced proliferation and decreased stimulation index in fish exposed to ammonia. Such effects may have important implications with respect to parasitization.

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REFERENCES

ALABASTER, J. S. & LLOYD, R. (1982). *Water Quality Criteria for Freshwater Fish*. EIFAC Report (FAO), 2nd Edn. Butterworths, London.

BANERJEE, S. & BHATTACHARYA, S. (1997). Histopathological changes induced by a chronic nonlethal levels of elsan, mercury and ammonia in the liver of *Channa punctatus* (Bloch). *Journal of Environmental Biology* **18**, 141–148.

BENNETT, A. F. (1987). Interindividual variability: an underutilised resource. In *New Directions in Ecological Physiology* (ed. Feder, M. E., Bennet, A. F., Burggren, W. W. & Huey, R. B.), pp. 147–169. Cambridge University Press, Cambridge, UK.

CENINI, P. (1984). The ultrastructure of leucocytes in carp (*Cyprinus carpio*). *Journal of Zoology* **204**, 509–520.

CLARK, K. (1993). *Toxicology of Water Pollutants*. Chemical Systems Group, CIT Shrivenham, Royal Military College, UK.

DEPLEDGE, M. H. (1990). New approaches in ecotoxicology: can inter-individual physiological variability be used as a tool to investigate pollution effects? *Ambio* **19**, 251–252.

DEPLEDGE, M. H. (1994). The rational basis for the use of biomarkers as ecotoxicological tools. In *Nondestructive Biomarkers in Vertebrates* (ed. Fossi, M. C. & Leonzio, C.), pp. 271–295. Lewis Publishers, Boca Raton, Florida.

DEPLEDGE, M. H., AMARAL-MENDES, J. J., DANIEL, B., HALBROOK, R. S., KLOEPPER-SAMS, P., MOORE, M. N. & PEAKALL, D. B. (1993). The conceptual basis of the biomarker approach. In *Biomarkers : Research and Application in the Assessment of Environmental Health* (ed. Peakall, D. B. & Shugart, L. R.), NATO ASI Series h; Cell Biology, Vol. 68, pp. 15–29. Springer Verlag, Berlin.

DEZFULI, B. S., CAPUANO, S. & MANERA, M. (1998). A description of rodlet cells from the alimentary canal of *Anguilla anguilla* and their relationship with parasitic helminths. *Journal of Fish Biology* **53**, 1084–1095.

DHABHAR, F. S., MILLER, A. H., MCEWEN, B. S. & SPENCER, R. L. (1995). Effects of stress on immune cell distribution: Dynamics and hormonal mechanisms. *Journal of Immunology* **154**, 5511–5527.

HOOLE, D. (1997). The effects of pollutants on the immune response of fish: implications for helminth parasites. *Parassitologia* **39**, 219–225.

KHAN, R. A. & THULIN, J. (1991). Influence of pollution on parasites of aquatic animals. *Advances in Parasitology* **30**, 199–238.

KIRK, R. S. & LEWIS, J. W. (1992). The laboratory maintenance of *Sanguincola inermis* Plehm, 1905 (Digenea: Sanguinicolidae). *Parasitology* **104**, 121–127.

LINTON, T. K., REID, S. P. & WOOD, C. M. (1997). The metabolic costs and physiological consequences to juvenile rainbow trout of a simulated summer warming scenario in the presence and absence of sublethal ammonia. *Transactions of the American Fisheries Society* **126**, 259–272.

LLOYD, R. (1961). Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (*Salmo gairdneri* Richardson). *Journal of Experimental Biology* **38**, 447–455.

LLOYD, R. (1992). *Pollution and Freshwater Fish*. Fishing News Books, London.

LLOYD, R. & HERBERT, D. W. M. (1960). The influence of carbon dioxide on the toxicity of un-ionised ammonia to rainbow trout (*Salmo gairdneri*). *Annals of Applied Biology* **45**, 521–527.

LLOYD, R. & ORR, L. D. (1969). The diuretic response by rainbow trout to sublethal levels of ammonia. *Water Research* **3**, 335–344.

MAGAUD, H., MIGEON, B., MORFIN, P., GARRIC, J. & VINDIMIAN, E. (1997). Modelling fish mortality due to urban storm run-off: interacting effects of hypoxia and un-ionised ammonia. *Water Research* **31**, 211–218.

RICHARDS, D. T., HOOLE, D., LEWIS, J. W., EWENS, E. & ARME, C. (1994*a*). Ultrastructural observations on the cellular response of carp *Cyprinus carpio* L., to eggs of the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae). *Journal of Fish Diseases* **17**, 439–446.

RICHARDS, D. T., HOOLE, D., LEWIS, J. W., EWENS, E. & ARME, C. (1994*b*). Changes in cellular composition of the spleen and pronephros of carp *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae). *Diseases of Aquatic Organisms* **19**, 173–179.

RICHARDS, D. T., HOOLE, D., LEWIS, J. W., EWENS, E. & ARME, C. (1996). Stimulation of carp *Cyprinus carpio* lymphocytes *in vitro* by the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae). *Diseases of Aquatic Organisms* **25**, 87–93.

THURSTON, R. V., RUSSO, R. C., LUEDTKE, R. J., SMITH, C. E., MEYN, E. L., CHAKOUMAKOS, C., WANG, K. C. & BROWN, C. J. D. (1984). Acute toxicity of ammonia to rainbow trout. *Transactions of the American Fisheries Society* **113**, 56–73.

TRUSSELL, R. P. (1972). The percent un-ionised ammonia in aqueous ammonia solutions at different pH levels and temperatures. *Journal of the Fisheries Research Board of Canada* **29**, 1505–1507.

WESTER, P. W., VETHAAK, A. D. & VAN MUISWINKEL, W. B. (1994). Fish as biomarkers in immunotoxicology. *Toxicology* **86**, 213–232.

ZELIKOFF, J. T., ENANE, N. A., BOWSER, D., SQUIBB, K. S. & FRENKEL, K. (1991). Development of fish macrophages as a model for higher vertebrates in immunotoxicological studies. *Fundamental and Applied Toxicology* **16**, 576–589.