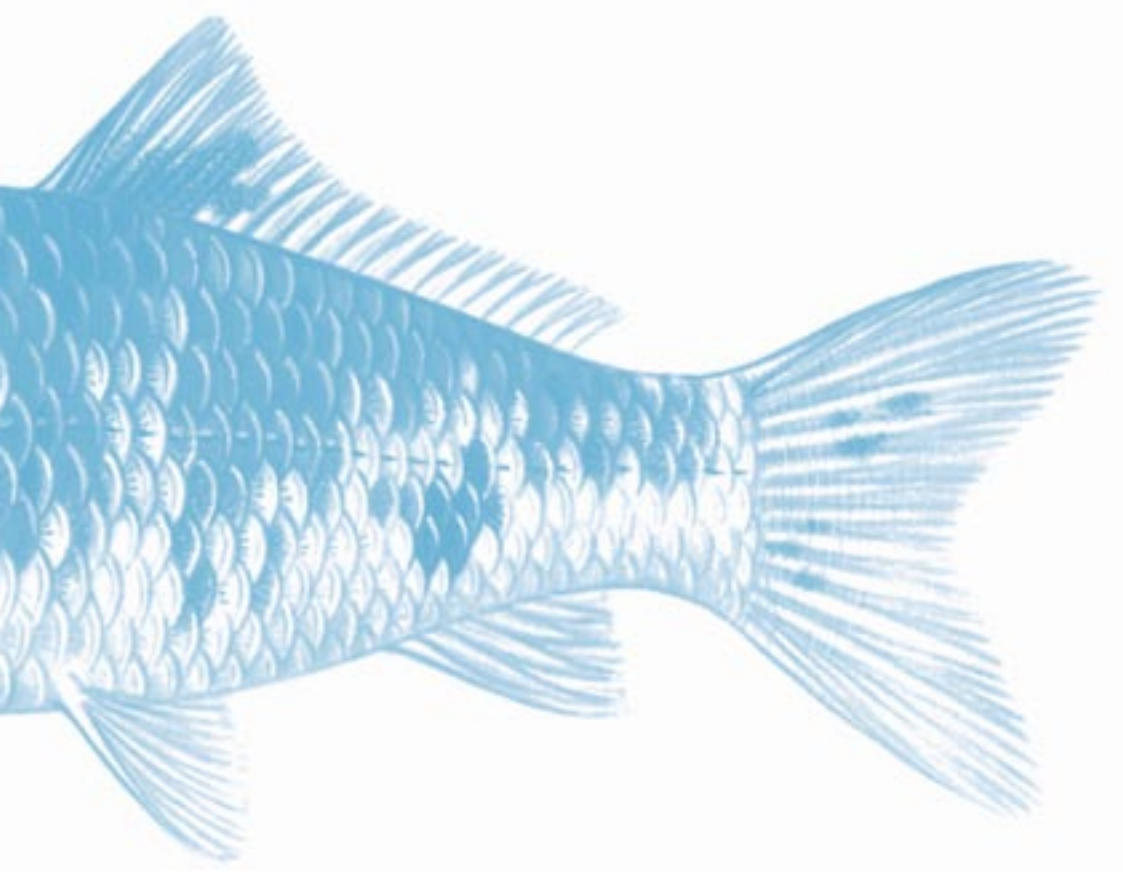
# *FISH VETERINARY JOURNAL*

**The Journal of the Fish Veterinary Society**

**Issue Number 8 • March 2005**



CONTENTS

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The *Fish Veterinary Journal* is published by the Fish Veterinary Society and provided free to the Society's members. Additional copies can be obtained from the editorial department for £15 (UK) and £25 (overseas).

The FISH VETERINARY SOCIETY was formed in 1990, with the intention of bringing together veterinarians with an interest in fish, so that they may benefit from mutual experiences and discussions, and help to advance the veterinary care and welfare of fish.

The Society provides:

- two scientific meetings, held annually
- publication of the *Fish Veterinary Journal*
- publication of policy documents on fish health and welfare
- political lobbying and representation on behalf of the members' interests



**MEMBERSHIP** of the Fish Veterinary Society is open to members of the Royal College of Veterinary Surgeons, although the Society will consider applications from overseas veterinarians and those with an appropriate interest / degree as set out in the Constitution. Currently membership costs £50 per annum. Veterinary students and suitably qualified non-veterinary graduates may become associate (non-voting) members of the Society; veterinary students pay a half-yearly fee until they graduate, at which point they will become full members if they so wish. Enquiries regarding membership of the Fish Veterinary Society should be addressed to the Honorary Treasurer – see the application form at the back of this Journal.

## Notes for contributors

The *Fish Veterinary Journal* invites contributions from members and other professional colleagues and is keen to publish original research, review articles and clinical case histories on all aspects of fish health. Letters, book reviews and other comment on relevant topics are also welcomed.

Scientific articles submitted to, or published in, other refereed journals will not be considered for publication. Papers and short communications submitted for publication are subject to peer review. The editor has the final decision on publication and if accepted, the copyright becomes the property of the Fish Veterinary Society.

Manuscripts and all communications should be sent to J. McArdle, 119 Park Drive Ave, Castleknock, Dublin 15, Ireland. Manuscripts should be submitted in duplicate, typewritten using a Times or Roman font (double line spaced) on one side of A4 paper with wide margins. Scientific articles may also be submitted as a Word document on 3½" diskette (MS-DOS format) or by email to mcardlej@eircom.net. The Journal cannot accept responsibility for loss or damage of manuscripts.

**Format:**

Papers should be headed with the full title, which should describe accurately the subject matter. The initials and surnames of the authors, with full postal addresses should follow. Each paper should have a self-contained summary (maximum of 150 words), which embodies the main conclusions.

Abbreviations should be avoided. Where they must be used, the word or phrase must be given on the first occasion, e.g. infectious pancreatic necrosis (IPN). All units of measurement should be given in the metric system and temperatures in degrees Celsius. Blood biochemistry values should be expressed in standard SI units. Medicinal products should be referred to by their generic name followed by proprietary name and manufacturer in brackets when first mentioned, e.g. amoxycillin (Vetremox®, Vetrepharm). The full Latin name for each species should appear at least once when mentioned in the text.

**Length of papers:**

Papers should be concise. As a guide, the maximum length for scientific articles is 3,000 words; for review articles up to 4,000 words; for short communications and clinical case reports up to 1,500 words.

**Tables and illustrations:**

The minimum number of figures necessary to clarify the text should be included and should contain only essential data. Tables must be typewritten on separate sheets and numbered. Illustrations should be drawn in black ink on white paper and should be suitable for direct photographic reproduction.

Legends should be typed on a separate sheet. Photographs should be clear and sharp, and in colour where possible (transparencies should be accompanied by one set of prints). Photomicrographs must state magnification and stain technique. Each illustration or photograph should bear the author's name and figure number in pencil (or on a label) on the back and an arrow used to identify the top edge. All photographs will be printed in black & white but may be reproduced in colour at the expense of the author(s).

**References:**

Only papers closely related to the author's work should be mentioned. These should be stated in chronological order in the body of the text and should be listed in alphabetical order and include the full title thus:

Hanson, L.A. & Grizzle, J.M. (1985) Nitrite-induced predisposition of channel catfish to bacterial disease. *Progressive Fish-Culturist* **47**, 98–101

Morrison, C.M., Cornick, J.W., Shum, G. & Zwicker, B. (1984) Histopathology of atypical *Aeromonas salmonicida* infection in Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases* **7**, 477–494

Roberts, R.J. (1993) Motile aeromonad septicaemia. In: *Bacterial Diseases of Fish*. (eds. V. Inglis, R.J. Roberts & N.R. Bromage). Blackwell Scientific Publications, Oxford. pp143–155

If three or more authors are quoted, then all must be listed in the references and should be written as, for example, 'Morrison et al 1984' in the body of the text.

Personal communications should be cited as such.

**Miscellaneous:**

A brief personal profile of academic achievements and the current position of the author(s) is also required as a footnote (maximum of 100 words) for scientific articles.

The *Fish Veterinary Journal* is covered by the CAB abstracts database.

# President's Reflections

**Lydia Brown**
Pharmaq Ltd.,Unit 15, Sandleheath Industrial Estate, Fordingbridge, Hants SP6 1PA

I would like to use this opportunity to inform FVS members and those who read the Fish Veterinary Journal of some of the activities carried out by FVS during 2004. The Spring meeting held in conjunction with the Glasgow Show in May 2004 was a well-attended event and covered a wide range of subjects. This was a joint meeting with the Scottish Fisheries Research Service and we used the meeting to discuss items that both parties could work on together in the future. These included future joint meetings and, over the long term, compilation of information on a readily accessible database.

Earlier this year I tried to circulate the new draft EU fish health directive for comment but got very few replies. This may have been because some members didn't receive the attachment I sent. My concern is that not all members may be on the list serve. Please remember that you must actively sign up for the list serve and this cannot be done by anyone else on your behalf. If you don't know how to do this please contact Chris Walster who can assist you. I hope that we can all make an effort to improve electronic communication between members. An up to date list serve is vital for us all to communicate effectively with each other.

The Fish Veterinary Society is a Division of the British Veterinary Society (BVA) and is represented on the Council of BVA. Keith Treves Brown does a very good job on our behalf making our views known. Keith would welcome support from any members who are interested in BVA affairs to help compile responses to Council papers. Keith lets the committee know in advance what the major issues are at BVA Council then reports back to FVS. We are often asked to respond to consultation documents etc. from a number of sources. If you are interested in helping with this please contact Fiona MacDonald our Hon. Secretary.

The Aquaculture Health Joint Working Group (AHJWG) is a body representing the aquaculture industry, DEFRA, FRS, other industry representatives and also FVS. The group meets a few times a year in Scotland and I represent FVS on this important body. I have attended one meeting to date and I circulate my minutes to the committee immediately after the meeting as well as

the official minutes when they become available. If any FVS member feels they should be more widely circulated please let me know. The most recent document to pass through the AHJWG is a draft code of practice for Scottish finfish aquaculture. It will shortly go out to public consultation. FVS commented on the draft document earlier this year but if individuals wish to take the opportunity it will soon be available to do so.

Fish Veterinary Society has taken a keen interest in the proposal to legislate for a new Veterinary Surgeons Act. Paraprofessionals' training, accreditation and professional conduct issues and how an RCVS of the future will work under a new Act is currently getting much discussion by a special working party of the RCVS. There will be further consultation within the profession and the working party wish to meet with representatives of paraprofessionals in the fish world.

I would like to especially acknowledge the great work done by Edward Branson, ably assisted by Chris Walster, in organising the Fish Welfare Meeting held on 23rd and 24th of November 2004 in Edinburgh. This was a most successful and well-attended meeting, which covered the entire broad area of fish welfare. It is planned to publish the papers presented at the meeting as a book, which will serve as a work of reference for years to come. This meeting is one of the most significant projects undertaken by FVS to date.

Finally, I want to thank all the FVS committee members and others on your behalf for all the hard work they put into running FVS. Much work goes on quietly and unnoticed behind the scenes so a big thanks to Pete Southgate, Edward Branson, Fiona Macdonald, Chris Walster and John McArdle as well as David Sutherland who has taken over production of the newsletter. Both John and David suffer from a lack of information and papers coming in from the membership. If you have an item, scientific paper or interesting clinical case which could be published or which other members might find interesting, then please contact either of them.

All the above named people and many unnamed as well who assist FVS are extremely busy people but without their efforts none of the work of the Society could be done. Please give them your support and help to ease the load. Remember this is your society. Let us know what you would like to see happen in the future.

# Editor's Comments

**John McArdle**
**119, Park Drive Avenue, Castleknock, Dublin 15, Ireland**

The FVS highlight of the year for 2004 was surely the Fish Welfare Meeting held in Edinburgh. To ensure the maximum dissemination of the information presented at that meeting it is planned to bring the papers out in the form of a book to be published by an international publishing house. This publication will certainly be worth waiting for. This edition of FVJ contains many of the papers presented at our more usual previous spring and autumn meetings together with scientific and review papers submitted independently of these meetings. Together with this issue of FVJ, I have also included a corrected version of Scott Peddie's valuable fish health economics papers from the previous issue since it contained some serious errors in the figures. *Mea culpa*! My sincere apologies to Scott and his co-author for these errors and I hope this goes some way to put matters right.

I am particularly pleased to include a couple of clinical papers from Sophie St. Hilaire and others from CEFAS, which can be considered work in progress. I am grateful to Denis Glasscock of CEFAS for giving permission so readily for publication of these preliminary findings. I like to think that this type of material is always of interest to those of our members who are involved in the investigation of disease outbreaks in farmed and wild fish on a day-to-day basis. I would like to see more of this type of paper submitted to the Journal.

This issue also contains an interesting personal view from Andrew Grant on control of saprolegniasis. This is based on a paper given by Andrew to an FVS meeting and contains all the material contained in that presentation plus a lot more useful information. This is an extremely important and often intractable disease as any fish veterinarian who has had to struggle with a serious outbreak will testify and this paper will be often referred to for assistance.

Áine Maxwell's paper on the role of the expert witness contains a fund of useful, quite compressed information on this topic. On a personal note, I remember in my first few months following qualification being subpoenaed to appear as an expert witness in a Dublin court in a cruelty case. I like to think I performed reasonably well but having a copy of Áine's paper to hand

then would have helped greatly. Although I brought photographs along, I did not even know that photographs were admissible as evidence. The judge's horror on seeing my photographs showing the pathetic state of the animal involved undoubtedly resulted in a successful prosecution.

Scott Peddie and Chris secombes have submitted a most useful review paper on the current state of research into immunostimulants in relation to fish. For some veterinarians such as myself, the subject of immunostimulants and their usefulness is something of a mystery and this paper will certainly act as a good introduction to the subject and the accompanying bibliography alone will be a useful source of reference. The paper deliberately avoids tricky legal questions about the medicinal classification of these compounds and concentrates on the available scientific information.

I was also delighted to receive Charlie's McGurk's paper on PKD. Anyone who saw his presentation at our FVS meeting will have been captivated by the sheer brilliance of his use of video and other visual techniques in his presentation. He has surely elevated the standard of presentation to a new level!

It is not possible to mention all the authors who have submitted papers to FVJ on this occasion but to those not specifically mentioned, be assured that their papers are all of an excellent standard and should be of great interest to our members and other readers. I would also like to thank our referees who carry out their invaluable work anonymously and generously.

Editing this issue has been particularly challenging for me for reasons I won't go into, but, regrettably, this has delayed publication a little. My sincere apologies to one and all for that delay.

As always, I must thank our advertisers for supporting FVJ. Obtaining advertising is becoming increasingly difficult as companies look more closely at their costs and have to spread scarce resources ever more thinly. Finally, I would like to thank Mike Williams of Akalat Publishing, whose patience and unfailing helpfulness makes the task of producing the Journal possible.

REVIEW PAPER

*Fish Veterinary Journal (2005) (8) 1–31*

# An Overview of Fish Immunostimulant Research

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*Abstract*

*Immunostimulants are increasingly viewed as an integral part of disease prevention and management in modern aquaculture. A large number of putative immunostimulants have been investigated in fish and include killed pathogens and their products, fungal β-glucans, chitin, unidentified animal extracts, synthetic chemicals, plant molecules, nutrients, RNA and host/animal molecules. Furthermore, following recent success in the cloning and sequencing of a range of immunologically important genes such as interleukin-1 beta (IL-1β), a new generation of immunostimulants can be produced as recombinant proteins or peptides, thus enabling the characterisation of their effects in homologous systems. This paper reviews the current status of immunostimulant research in aquatic animals.*

## Introduction

An immunostimulant is defined as a 'chemical, drug, stressor, or action that elevates the non-specific defence mechanisms or the specific immune response.' (Anderson 1992). Numerous naturally derived and artificially synthesised products have demonstrated a broad range of immunostimulatory effects and can be administered either alone, or combined with a vaccine preparation to enhance its efficacy.

Recent interest in the application of immunostimulants has been heightened as a result of the increasing economic impact of infectious disease. This problem is exacerbated by the lack of chemotherapeutic agents that are approved for use in food fish. Furthermore, the accumulation of substances in the environment has to be considered, as does the possibility of an increase in the prevalence of antibiotic resistant strains of bacteria in the aquatic envi-

ronment surrounding farm units. Moreover, the prophylactic application of immunostimulants prior to situations where there is an increase in disease risk (Anderson 1992; Raa 1996), including transport and handling, a change in environmental conditions and smoltification in salmonids is gaining acceptance (Raa 1996). Thus, immunostimulant use is increasingly seen as an integral part of the multifaceted approach to disease control.

The aim of this paper is to review the major categories of immunostimulants that have been investigated for use in aquatic animals.

## A Review of the Properties and Actions of Fish Immunostimulants

There are a plethora of products that have immunostimulatory properties in fish. Such products can be classified as killed pathogens and their products, synthetic chemicals, plant molecules, host/animal molecules and nutrients. This remainder of this paper reviews each of these categories of immunostmulants in detail.

### a) Killed Pathogens and their Products

#### *Freund's Complete Adjuvant / Incomplete Freund's Adjuvant*

Freund's complete adjuvant (FCA) consists of a preparation of mycobacterial cell walls suspended in mineral oil (Moritana *et al.* 1992; Raa 1996). Kajita *et al.* (1992) injected rainbow trout (*Onchorhynchus mykiss)* with FCA; survival following *Vibrio anguillarum* challenge, respiratory burst activity and phagocytic ability of head kidney leucocytes were all enhanced significantly relative to controls. More recently, Brenek *et al.* (2002) demonstrated that a combination of FCA and excretory-secretory products of *Trypanosoma danilewskyi* injected into goldfish conferred significant protection against infection.

Incomplete Freund's Adjuvant (IFA) contains the same combination of emulsifying agents and physiological saline as FCA, although it does not contain the *Mycobacterium* component (Anderson 1992). Commercial usage of FCA and IFA is precluded as a consequence of the granulomas and open lesions that may develop at the site of injection (Anderson 1997).

#### *Cholera Toxin /Cholera Non-Toxic B sub-unit*

Hebert *et al.* (2000) demonstrated that it is possible to modulate the serum antibody response to intraperitoneally administered antigens by conjugation to adjuvants such as cholera toxin (CT) and cholera non-toxic B sub-unit

(CTB). Increased adjuvant effects were observed by encapsulating IgG-CT, and by the addition of CT to the CTB-antigen treatments. Merino-Contreras *et al.* (2001) reduced mortality rates in spotted sand bass (*Paralabrax maculatofasciatus*) by orally administering MCBP (an extracellular lectin from *Aeromonas veronii*) covalently linked with CTB.

***Muramyl Dipeptides***

Muramyl dipeptide (MDP) is a biologically active mycobacterial peptidoglycan derivative (N-acetyl-muramyl-L-alanyl-D-isoglutamine) (Anderson 1992; Secombes 1994). It has been shown to augment disease resistance in fish (Matsuo & Miyazono 1993; Itami *et al.* 1996), in addition to enhancing respiratory burst, phagocytic and migratory activity of head kidney phagocytes at 1-2 days post injection (Kodama *et al.* 1993).

***Lipopolysaccharides***

Lipopolysaccharide (LPS) preparations consist of O-antigens and endotoxins extracted from Gram-negative bacteria such as *Salmonella typhimurium* or *Escherichia coli* (Anderson 1992; Secombes 1994). The biological activity of LPS is a consequence of both the lipid moiety and core polysaccharide (Raa 1996).

In appropriate doses, LPS demonstrates immunostimulatory properties both *in vivo* and *in vitro* (Anderson 1992). With respect to the former, Hine & Wain. (1988) injected eels (*Anguilla australis)* with 1mg LPS 100g body weight$^{-1}$, that resulted in enhanced neutrophil production. In plaice (*Pleuronectes platessa*), LPS induced a significant increase in peritoneal leucocyte numbers in addition to enhanced splenic macrophage migratory activity after injection of 500µg LPS for 5 days (MacArthur *et al.* 1984). *Edwardsiella tarda*-derived LPS increased phagocytic activity and disease resistance when administered to eels (Salati *et al.* 1987), whilst in rainbow trout, LPS administration elicited an acute-phase hypoferremic response (Congleton & Wagner 1991). Furthermore, Kjerstad *et al.* (1998) demonstrated that LPS could be utilised as an agent to stimulate non-specific defence mechanisms in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, thus augmenting survival. Interestingly, a more recent experiment by Guttvik *et al.* (2002) showed that Atlantic salmon fry fed with LPS-coated feed (0.1% LPS) for 63 days had a reduced survival rate when challenged with a virulent strain of *A. salmonicida*.

In the context of *in vitro* LPS application, lymphocyte proliferation has been observed in salmonid cell cultures in response to LPS (Warr & Simon 1983;

Reitan & Thuvander 1992). Moreover, in channel catfish (*Ictalurus punctatus)*, monocytes incubated with 10μg $ml^{-1}$ LPS for 48h induced production of IL-1 like compounds (Clem *et al.* 1985). *In vitro* administration of LPS enhanced respiratory burst, phagocytic and bactericidal activity of head kidney macrophages isolated from Atlantic salmon (Jørgensen 1994; Solem *et al.* 1995). Salmon head kidney macrophages grown in the presence of LPS showed increased production of lysozyme in the culture supernatants, concomitant with an accumulation of lysozyme gene transcript in stimulated cells (Paulsen *et al.* 2001). Indeed, an optimal response was observed at 10μg $ml^{-1}$ LPS. Campos Pérez *et al.* (1997) demonstrated that synergy of LPS and recombinant human TNF-α enhanced respiratory burst activity in trout, concomitant with an increased ability to kill *Renibacterium salmoninarum*. Moreover, inducible Nitric Oxide Synthase (iNOS) activation has been documented in goldfish macrophages as a consequence of synergistic application of LPS and macrophage activating factors (Neumann *et al.* 1995). Lastly, Zou *et al.* (2000) reported IL-1β expression in rainbow trout leucocytes treated with 0.1 to 5μg LPS $ml^{-1}$.

***Formyl Peptides***

Formyl-methionyl-leucyl-phenylalanine (FMLP) is an artificially synthesised molecule based on a bacterially derived peptide product (Nash *et al.* 1986) that has been shown to act as a leucocyte chemoattractant in plaice (Nash *et al.* 1986) and red sea bream (*Pagrus major*) (Watanabe *et al.* 1997).

***Bacille Calmette Guérin (BCG)***

BCG is a cell wall preparation of *Mycobacterium bovis*; the most immunoactive component is the mycolic acid-aribinogalactan-mucopeptide complex (Raa 1996). Grayson *et al*. (1987) demonstrated increased resistance to ERM (Enteric Redmouth disease) in trout intraperitoneally injected with an ERM vaccine containing a BCG adjuvant.

***Fungal β-Glucan /Chitin***

Fungal β-glucans are polyglucoses which consist of a linear backbone of β-1,3 linked D-glucopyranosyl residues with varying degrees of branching from the C6 position (Bohn & Bemiller 1995). The branches contain either single or multiple glucose molecules linked with β-1,3 or β-1,6 linkages (Raa 1996). Chitin is an insoluble β-1,4-linked polymer of N-acetyl-D-glucosamine. Although not a glucan, chitin is a common constituent of fungal cells walls and will therefore be discussed in this section.

The glucans that have been studied with respect to their effects in fish can be categorised as derivatives of either: the yeast *Saccharomyces cerevisiae* (yeast glucan, e.g. Macrogard), the mushroom *Lentinus edodes* (lentinan), the mycelial fungi *Schizophyllum commune* (schizophyllan, VST) or the mycelial fungi *Sclerotium glucanicum* (scleroglucan). These glucans differ in molecular weight, degree of polymerisation, structural configuration and solubility (Raa 1996). Each of the above will be discussed in detail in the text that follows. Chitin will be reviewed at the end of the section.

*Yeast Glucan*

It has long been known that a crude preparation of yeast cell walls, zymosan, has immunostimulatory properties (Raa 1996). More recently, it has been shown that Atlantic salmon macrophages possess receptors that specifically recognise yeast derived β-glucan (Engstad & Robertsen 1994), whilst a zymosan receptor has been identified in catfish neutrophils (Ainsworth *et al*. 1994).

There are a number of reports that document enhanced protection of fish following yeast glucan administration. Species studied include Atlantic salmon (*Salmo salar*)(Robertsen *et al.* 1990), brook trout (*Salvelinus fontinalis*) (Anderson & Siwicki 1994) and channel catfish (Chen & Ainsworth 1992). Indeed, yeast glucan therapy has been shown to be effective in augmenting fish defence against bacterial, protozoal (Raa 1996) and more recently, viral infections (LaPatra 1998).

Injection of yeast glucans augments levels of lysozyme mediated lytic activity in Atlantic salmon (Engstad *et al.* 1992), rainbow trout (Jørgensen *et al.* 1993) and turbot (*Scophthalmus maximus*) (Santarem *et al.* 1997) serum. A recent study has demonstrated that Atlantic salmon head kidney macrophages cultured in the presence of particulate yeast β-glucan showed increased production of lysozyme in culture supernatants, in addition to accumulation of lysozyme gene transcript in stimulated cells (Paulsen *et al.* 2001). Indeed, β-Glucan showed an approximate linear dose-response curve between 1 and 250μg $ml^{-1}$. Finally, complement activity is also stimulated in Atlantic salmon injected with β-Glucan, although the temporal dynamics of the response is different to that observed for lysozyme (Engstad *et al.* 1992).

With respect to cellular responses in fish treated with yeast glucan, increases in phagocytic ability (Chen & Ainsworth 1992; Kodama *et al.* 1993; Ortuno *et al.* 2002), respiratory burst activity (Jørgensen *et al.* 1993; Brattgjerd *et al.* 1994; Ortuno *et al.* 2002) and bactericidal activity (Chen & Ainsworth 1992;

Jørgensen *et al.* 1993) have been documented. More specifically, increased blood neutrophil numbers and nitroblue tetrazolium (NBT) positive cells were recorded in rainbow trout following yeast β-glucan administration (Jeney & Anderson 1993). Jørgensen. (1994) injected a 1% suspension of M-glucan into Atlantic salmon, which resulted in maximal accumulation of macrophages and neutrophils in the peritoneum 2 days post injection; this inflammatory response is important for effective functioning as an adjuvant.

Oral administration of yeast β-glucan to rainbow trout results in significantly enhanced phagocytic ability, myeloperoxidase activity, oxidative radical production and total Ig levels. Moreover, this was correlated with enhanced protection against *A. salmonicida* challenge (Siwicki *et al.* 1994). Yoshida *et al.* (1995) administered β-glucan orally to catfish for 45 days and recorded increased numbers of neutrophils in the peripheral blood, whilst in turbot, Toranzo *et al.* (1995) recorded augmented levels of NBT positive cells. In the latter however, the enhanced immune parameter did not correlate with enhanced resistance against *Enterococcus* sp. challenge. In rainbow trout, Verlhac *et al.* (1998) failed to elicit increased respiratory burst activity, pinocytosis or blood lysozyme levels in fish fed dietary yeast β-glucan. A similar lack of immunostimulation was noted by de Baulny *et al.* (1996), although in this case the target species was turbot; yeast β-glucan did not significantly affect plasma lysozyme or complement activity in peripheral blood.

*Lentinan*

Lentinan has a molecular weight of $5x10^5$ Da and has two glucose branches for every five β-1,3-glucosyl units in the backbone (Raa 1996). Lentinan injected into carp (*Cyprinus carpio*) at a dose of 2-10mg kg body weight$^{-1}$ enhanced resistance to experimental *E. tarda* challenge; this was correlated with increased phagocytic activity of pronephros leucocytes (Yano *et al.* 1989; 1991).

*Schizophyllan*

Schizophyllan is an extract from the fermentation broth of the basidiomycete fungus *Schizophyllum commune* (Raa 1996). Administration of schizophyllan has been shown to augment disease resistance in prawns (*Penaeus japonicus*) (Itami *et al.* 1994), carp (Yano *et al.* 1989; 1991), coho salmon (*Onchorhynchus kisutch*) (Nikl *et al.* 1991) and yellowtail (*Seriola quinqueradiata*) (Matsuyama *et al.* 1992). In yellowtail, injection of 10mg schizophyllan kg body weight$^{-1}$ facilitated enhanced complement and lys-

ozyme levels (Matsuyama *et al.* 1992). Augmented phagocytic activity of carp pronephros cells was also recorded by Yano *et al.* (1989) subsequent to schizophyllan application.

A commercial schizophyllan preparation, VitaStim-Taito administered in feed has been shown to increase disease resistance in salmonids (Nikl *et al.* 1992) and catfish (Ainsworth *et al.* 1994). Moreover, at a 0.1% inclusion level in feed, Ainsworth *et al.* (1994) recorded significantly enhanced antibody titres against *E. ictaluri*. With respect to non-specific immune immunostimulation, a recent study in the black tiger shrimp (*Penaeus monodon*), showed that in-feed administration of VitaStim (2.0g $kg^{-1}$) resulted in enhanced haemocyte phagocytic activity, cell adhesion and superoxide anion production (Chang *et al.* 2000).

*Scleroglucan*

Scleroglucan is an extract of the basidomycete fungus *Sclerotium glucanum* which like schizophyllan, has β-1,6 branches at every third glucose in the β-1,3 backbone (Raa 1996). It has been demonstrated that scleroglucan enhances disease resistance in carp (Yano *et al.* 1991), grass carp (*Ctenopharyngodon idella*) (Wang & Wang 1997), tilapia (*Tilapia* spp) (Wang & Wang 1997) and yellowtail (Matsuyama *et al.* 1992). Moreover, Matsuyama *et al.* (1992) correlated this increased disease resistance with enhanced levels of lysozyme and complement activity in yellowtail treated with scleroglucan. Yano *et al.* (1989) documented increased phagocytic activity of pronephros leucocytes, whilst Wang & Wang (1997) recorded an increase in the number of blood neutrophils and glass-adherent NBT positive cells in scleroglucan injected tilapia and grass carp.

*Chitin*

Chitin (poly [1→4]-β-N-acetyl-D-glucosamine) has been shown to have a stimulatory effect on the innate immune response of rainbow trout (Sakai *et al.* 1992) and gilthead seabream (*Sparus aurata* L.) *in vivo* (Esteban *et al.* 2000; Esteban *et al.* 2001). Such effects include an increase in natural haemolytic complement activities, cellular respiratory burst and phagocytosis (Esteban *et al.* 2000, 2001).

**b) Synthetic Chemicals**

***Levamisole***

Levamisole is a synthetic compound with the chemical name 2,3,5,6-tetrahy-

dro-6-phenylimidazo[2,1-b] thiazole; it was initially developed to combat parasitic infections in ruminants (Raa 1996).

Injection of 0.5mg levamisole kg body weight$^{-1}$ increases natural killer cell activity, complement levels and bactericidal activity in trout, in addition to enhancing resistance to *V. anguillarum* challenge (Kajita *et al.* 1990). Siwicki (1989) recorded enhanced phagocytic, myeloperoxidase and migratory activity in carp neutrophils; the effective dosage regime consisted of injecting fish with 5-10mg levamisole kg body weight$^{-1}$ on 3 occasions 3 days apart. Oral administration of levamisole at 3-day intervals over a 15-day time course was also effective in enhancing phagocytic activity (Siwicki 1989). More recently, Findlay *et al.* (2000) exposed Atlantic salmon to 2.5mg l$^{-1}$ levamisole as a bath treatment for 2h. After 2 weeks, non-specific immunostimulation was demonstrated by increases in phagocytic activity, increased levels of the reactive oxygen intermediate superoxide anion, and an increased lytic activity of both the mucus and serum (Findlay *et al.* 2000).

*In vitro* administration of levamisole in trout spleen cells has been shown to increase phagocytic activity and NBT reduction at a dose rate of 5µg ml$^{-1}$ culture medium over a 10-day period (Siwicki *et al.* 1990). This response was augmented by administering levamisole in combination with the *Yersinia ruckeri* O-antigen (Siwicki *et al.* 1990).

***FK-565***

FK-565 (heptanoyl-y-D-glutamyl-(L)-mesodiaminopimelyl-(D)-alanine) is a synthetic lactoyl tetrapeptide based on a *Streptomyces olivaceogriseus* extract (Anderson 1992; Secombes 1994; Raa 1996). A single intraperitoneal injection of 1mg FK-565 kg body weight$^{-1}$ in rainbow trout resulted in significantly enhanced phagocytic and bactericidal activity, whilst *in vitro* administration of FK-565 at 20 and 40µg ml$^{-1}$ for 20h enhanced respiratory burst and spreading activity in leucocytes (Kitao & Yoshida 1986). FK-565 was also found to be an effective adjuvant when injected in conjunction with *Y. ruckeri* and *A. salmonicida*; in treated rainbow trout, a significant increase in both plaque-forming cells and humoral antibody titers occurred (Kitao *et al.* 1987).

***ISK***

ISK is a short chain polypeptide fish by-product (Anderson 1992; Secombes 1994). Intraperitoneal injection of this compound results in enhanced neutrophil and phagocytic activities, either when given alone or in an *A. salmonicida* O-antigen preparation (Anderson & Jeney 1992).

***RNA***

Injection of double stranded RNA (dsRNA) induces expression of the Mx gene in trout and salmon (Trobridge *et al.* 1997; Robertsen *et al.* 1997; Jensen *et al.* 1998). The resultant Mx proteins play an important role in virus inhibition. In salmon, Jensen *et al.* (1998) documented increased expression of the Mx genes for 14 days subsequent to dsRNA injection; the pattern of expression was correlated with the degree of protection exhibited against ISA. Similarly, Schelkunov *et al.* (1997) injected dsRNA from phage or yeast that resulted in decreased mortality and morbidity of carp/trout challenged with spring viraemia of carp virus (SVCV) or VHSV.

***Nucleotides/Oligonucleotides***

Sakai *et al.* (2001) orally administered nucleotides from yeast RNA to carp on a daily basis for 3 days and noted enhanced phagocytic and NBT activities in kidney leucocytes, concomittant with augmentation of serum complement and lysozyme levels. In Atlantic salmon, Burrells *et al.* (2001a,b) showed that supplementary dietary nucleotides added to feed at 0.03% inclusion rates enhanced vaccine efficacy, in addition to increasing resistance to experimental challenge with *V. anguillarum*, ISAV, *Piscirickettsia salmonis* and *Lepeoptheirus salmonis*. Recently, Jørgensen *et al.* (2001) demonstrated that synthetic oligodeoxynucleotides containing unmethylated CpG motifs induce IL-1β expression and production of interferon-like cytokines in rainbow trout head kidney macrophages *in vitro*. CpG treatment can also enhance disease resistance and leucocyte proliferation (Carrington, pers comm, Scottish Fish Immunology Research Centre, University of Aberdeen).

**c) Plant Molecules**

***Quil-A***

Bath administration of Quil-A saponin with *Yersinia ruckeri* vaccine enhances *in vitro* bactericidal activity in trout (Grayson *et al.* 1987), whilst oral administration stimulates leucocyte migration in yellowtail (Ninomiya *et al.* 1995). Ashida *et al.* (1999) fed Japanese flounder (*Paralichthys olivaceus*) a combination of quillaja saponin and formalin killed *Edwardsiella tarda* cells prior to an *E.tarda* challenge. Fish receiving the immunostimulant showed higher survival rates than the control group.

***Glycyrrhizin***

A glycosylated saponin, Glycyrrhizin, is an anti-inflammatory agent that contains glycyrretinic acid (Zhang *et al.* 1990). In the rainbow trout, *in vitro*

administration results in stimulation of macrophage respiratory burst activity and lymphocyte proliferation (Jang *et al.* 1995).

***Algal Extracts***

In the piscine model, extracts from the macro-algae *Laminaria hyperborean* have been shown to enhance spreading, pinocytosis, intracellular production of superoxide anion and acid phosphatase activity in Atlantic salmon head kidney macrophages (Dalmo & Seljelid 1995). In carp, sodium alginate enhances the migration of head kidney phagocytes to the site of injection, whilst concomitantly enhancing their phagocytic activity. Moreover, sodium alginate stimulates peritoneal leucocytes to produce chemotactic factors, in addition to augmenting the sensitivity of head kidney phagocytes to such factors (Fujiki & Yano 1997).

Miles *et al.* (2001) demonstrated that striped snakehead (*Channa striata*) injected intraperitoneally with 500µg Ergosan (Schering-Plough Aquaculture), a commercial preparation containing alginic acid, resulted in immunostimulation. More specifically, both serum and macrophages from treated fish had significant inhibitory effects on the germination and subsequent growth of *Aphanomyces invadans* (=*piscicida*), the causative agent of epizootic ulcerative syndrome, at 14 days post-injection. In trout, Peddie *et al.* (2002b) found that migration of leucocytes into the peritoneal cavity was stimulated following intraperitoneal administration of ≥1mg $ml^{-1}$ Ergosan. Moreover, a single dose of 1mg significantly augmented the proportion of neutrophils in peritoneal exudates at 1 day post-injection, degree of phagocytosis, respiratory burst activity and expression of IL-1β, IL-8 and TNF-α genes.

***Herbal Extracts***

Ponpornpisit *et al.* (2001) administered a 0.5% sodium chloride bath to guppies (*Poecilia reticulata*) in conjunction with the in-feed delivery of a Chinese herb mix, known as C-UPIII. Such a prophylactic treatment regime reduced the incidence of disease in fish experimentally challenged with the ciliated protozoan *Tetrahymena pyriformis*. An earlier study had documented the immunostimulatory effects of C-UPIII administered alone to tilapia (*Oreochromis niloticus*) (Chansue *et al.* 2000). Longambal *et al.* (2000) found that administering a leaf extract of *Ocinum sanctum* to tilapia resulted in stimulated antibody responses and neutrophil activity. In-feed delivery of the leaf extract also resulted in augmented antibody titres, in addition to enhancing disease resistance against *A. hydrophila*.

In trout, Peddie & Secombes (2003) documented the immunostimulatory effects of Chevimmun (Chevita GmbH), a fixed combination herbal product administered intraperitoneally. Chevimmun is composed of 3 plant extracts: *Echinacea angustifolia* radix original tincture (200ml), *Eupatorium perfoliatum* original tincture (90ml) and *Baptisia tinctoria* radix original tincture (10ml). Trout injected with a 75% Chevimmun preparation exhibited enhanced peritoneal leucocyte migration, phagocytosis and intracellular respiratory burst activity.

**d) Host/Animal Molecules**

***Cytokines***

Mammalian derived cytokines which have been investigated as potential immunostimulants in fish include interferon-γ (IFN-γ), IL-4, monocyte chemotactic protein (MCP), granulocyte-macrophage CSF, IL-1, IL-6, IL-8 and TNF-α (Anderson 1992; Ahne 1993; Jørgensen *et al.* 1998, 2000; Secombes 1994).

Studies by Graham & Secombes (1988) and Hardie *et al.* (1994) documented the release of macrophage activating factor(s) (MAF) from rainbow trout and Atlantic salmon leucocytes. Macrophage processes that were augmented by MAF included spreading, adherence, phagocytosis, acid phosphatase levels, respiratory burst and bactericidal activity (Secombes & Fletcher 1992). It was postulated that MAF was a T cell product consistent with an IFN-γ like molecule (Secombes 1994). Moreover, Hardie *et al.* (1994) recorded a synergistic effect of MAF combined with recombinant human TNF-α.

*In vitro* application of mammalian recombinant IL-1, IL-6 and TNF-α resulted in expression of serum amyloid A (SAA) mRNA in Atlantic salmon hepatocytes; SAA is an acute phase reactant involved in the inflammatory response (Jørgensen *et al.* 1998, 2000).

Recently, the predicted rainbow trout mature IL-1β peptide has been produced as a recombinant protein, thus allowing its immunostimulant activities to be characterised in fish (Secombes *et al.* 2001). *In vitro* studies have shown that trout IL-1β increases the expression level of IL-1β, COX-2 and MHC class II beta chain transcription, in addition to augmenting murine D10.G4.1 cell proliferation and trout head kidney leucocyte phagocytosis (Hong *et al.* 2001) and migratory activity (Peddie *et al.* 2001). Moreover, rIL-1β injected intraperitoneally in trout enhanced leucocyte migration, phagocytosis and IL-1β, COX-2 and lysozyme II gene expression, whilst resistance to *A. sal-*

*monicida* was augmented at early times (2 days) post-injection (Hong *et al.* 2003). Interestingly, a synthetically produced IL-1β derived peptide, known as P3, enhanced head kidney leucocyte phagocytosis and bactericidal activity *in vitro* (Peddie *et al.* 2002a), in addition to enhancing leucocyte migration (Peddie *et al.* 2001). *In vivo* effects include leucocyte migration to the site of injection, enhanced phagocytic activity of peritoneal leucocytes and reduced mortality following viral haemorrhagic septicaemia virus (VHSV) challenge (Peddie *et al.* 2003). In carp, Kono *et al.* (2002) injected the carp IL-1β gene and recorded increased lymphocyte proliferation, macrophage function and enhanced resistance to *A. hydrophila* challenge.

***Eicosanoids***

*In vitro* application of synthetic leukotriene $B_4$ has been shown to induce the migration of leucocytes from the dogfish *Scyliorhinus canicula* (Hunt & Rowley 1986) and the rainbow trout (Sharp *et al.* 1992; Secombes *et al.* 1994). More recent studies have focussed on elucidating the kinetics of leukotriene $B_4$ stimulation in teleosts (Farndale *et al.* 1999; Holland *et al.* 1999; Tafalla *et al.* 1999). Moreover, arachidonic acid, an important component in the eicosanoid generating pathway has been shown to enhance thrombocyte aggregation *in vitro* (Hill *et al.* 1999), in addition to increasing the survival of gilthead seabream (*S. aurata*) larvae subjected to handling stress (Koven *et al.* 2001).

***Dimerized Lysozyme (KLP-602)***

In nature, lysozyme is found as a monomer, whilst the dimer is known to be significantly less bio-active (Siwicki *et al.* 1996; 1998). Experimental studies in terrestrial animals demonstrated that dimerized lysozyme activates phagocytosis, stimulates interferon synthesis and modulates TNF production (Siwicki *et al.* 1998). In fish, Siwicki *et al.* (1998) documented enhanced NBT reduction, respiratory burst activity, potential killing activity of blood phagocytes and myeloperoxidase activity in neutrophils following 10 and 100μg KLP-602 kg body weight$^{-1}$ injections 3 times every 2 days. Moreover, lysozyme activity and total Ig levels were augmented, whilst mortality rates following *A. salmonicida* challenge were significantly reduced.

***Fermented Chicken Egg Products (EF203)***

Yoshida *et al.* (1993) investigated the immunomodulatory effects of immunoactive peptides present in the fermented chicken egg products, EF203, administered orally to rainbow trout. Chemiluminescent response of kidney

phagocytes following treatment was significantly increased, as was resistance to both natural and experimental beta-haemolytic streptococcal infection; the immunomodulatory effects were dose-dependent.

***Lactoferrin***

Lactoferrin is an iron-binding glycoprotein that consists of a single chain peptide with a molecular weight of 87kDa, with 2 iron binding sites per molecule (Sakai *et al.* 1993). Bovine lactoferrin administered orally to rainbow trout at a dose of 100mg kg body weight$^{-1}$ for 3 days resulted in an increase in phagocytic and chemiluminescent activities of pronephros cells. Moreover, this coincided with enhanced resistance to intraperitoneal challenges of *V. anguillarum* and *Streptococcus* sp. (Sakai *et al.* 1993). Lygren *et al.* (1999) conducted a study in which Atlantic salmon were given 140mg bovine lactoferrin kg$^{-1}$ feed for 19 days. Lysozyme activity in serum and head kidney, complement-mediated haemolysis and phagocytosis were all unaffected by this treatment, as was resistance to experimental infection with infectious salmon anaemia virus (ISAV) and *A. salmonicida*.

***Hormones/Neurotransmitters***

Watanuki *et al.* (2000) recorded enhanced respiratory burst and phagocytic ability of rainbow trout macrophages incubated *in vitro* with 10-100ng ml$^{-1}$ of β-endorphin isolated from chum salmon. Moreover, β-endorphin also increased the production of superoxide anion in phagocytic cells prepared from the kidney of carp (*C. carpio*) (Watanuki *et al.* 2000).

According to Muñoz *et al.* (1998), overnight priming of Mediterranean sea bass (*Dicentrarchus labrax* L.) macrophages *in vitro* with 5nM recombinant rainbow trout growth hormone (rtGH) results in a significant increase in phagocyte respiratory burst activity. Furthermore, *in vivo* application of chum salmon growth hormone for 5 days resulted in augmented pronephros leucocyte chemiluminescence (Sakai *et al.* 1995, 1998).

Hormones that function primarily in the regulation of skin pigmentation in teleost fish also have immunostimulatory effects *in vitro*. Harris & Bird (1998) showed that alpha-melanocyte stimulating hormone (α-MSH) significantly increased phagocytic activity in rainbow trout head kidney macrophages incubated with 1, 5 and 10nM of the hormone. In addition, melanin-concentrating hormone (MCH) also directly influences the activity of rainbow trout phagocytes at concentrations between 5 and 500nM (Harris *et al*. 1998). Hormones such as α-adrenergic (phenylphrine) and choliner-

gic (carbachol) receptor agonists applied to trout kidney leucocytes *in vitro* enhance respiratory burst activity. This response was also documented in leucocytes exposed to adrenocorticotropic hormone (ACTH) at 100μg $ml^{-1}$. (Bayne & Levy 1991; Flory & Bayne 1991).

Finally, Keles *et al.* (2002) fed trout with a diet containing 2, 5, 10 and 20ppm zeranol, a synthetic oestrogen compound, for a period of 21 days. Interestingly, phagocytic and bactericidal activity of harvested leucocytes was enhanced in a dose-dependent manner, thus demonstrating the immunostimulatory effects of this synthetic hormone.

***Unidentified Extracts***

Enhanced disease resistance has been documented in eels (*Anguilla anguilla*) receiving aqueous extracts of the tunicate *Ecteinascidia turbinate*, although the immunoactive component has yet to be isolated and characterised (McCumber *et al.* 1982; Sigel *et al.* 1983; Davis & Hayasaka 1984). Sakai *et al.* (1991) reported increased phagocytosis, respiratory burst activity and disease resistance in rainbow trout 5 days after an intraperitoneal injection of an Abalone (*Haliotis discus hannai)* extract; the dose administered was 20mg extract 100g body $weight^{-1}$. More recently, Bøgwald *et al.* (1996) demonstrated that a peptide fraction (500-3000Da) from Atlantic cod (*Gadus morhua* L.) muscle protein hydrolysate had immunostimulatory properties; 30-1000μg $ml^{-1}$ of the peptide increased *in vitro* respiratory burst activity in salmon macrophages in a dose dependent manner. Furthermore, intraperitoneal injection of the peptide fraction in Atlantic salmon (100mg kg body $weight^{-1}$) resulted in enhanced levels of superoxide anion production in head kidney leucocytes 2 and 4 days post-injection (Bøgwald *et al.* 1996).

**e) Nutrients as Immunostimulants**

Vitamin C, vitamin E and polyunsaturated fatty acids have been the most intensively studied factors in the context of nutritional immunology (Blazer & Wolke 1984; Li & Lovell 1985; Secombes 1994; Raa 1996). In terms of the former, a number of studies have reported an increase in immune activity (Cuesta *et al.* 2002; Sahoo & Mukherjee 2003) and disease resistance following *in vivo* administration (Navarre & Halver 1989; Waagbö *et al.* 1994; Verlhac *et al.* 1998; Sahoo & Mukherjee 2003). For example, Verlhac *et al.* (1998) documented vitamin C modulation of respiratory burst activity, pinocytic ability of pronephros phagocytes and blood lysozyme activity. With respect to vitamin C modulated antibody production, various reports have

been published which describe this phenomenon (Erdal *et al.* 1991; Waagbö *et al.* 1994; Verlhac *et al.* 1998). Vitamin E levels in the diet have an influence on the phagocytic activity of peritoneal macrophages in trout (Blazer & Wolke 1984), complement activity in Atlantic salmon (Hardie 1991) and natural cytotoxic activity in the seabream (Cuesta *et al.* 2001). Interestingly, Sealy & Gatlin (2002) combined megadoses of vitamin C (2,500mg $kg^{-1}$) and vitamin E (300mg $kg^{-1}$) in juvenile hybrid striped bass diets, although it failed to enhance resistance to *Streptococcus iniae* challenge. Enhanced n-3 polyunsaturated fatty acids have been shown to enhance key immune parameters and disease resistance in fish (Salte *et al.* 1988; Erdal *et al.* 1991; Waagbö 1994), although the extent of immunostimulation is dependent on the n-3:n-6 ration (Ashton *et al.* 1994; Bell *et al.* 1996).

Less intensively studied factors, such as dietary iron levels, soya protein and synthetic carotenoids have been shown to modulate the piscine immune response. Lim *et al.* (2000) showed that macrophage migration in the presence or absence of *Edwardsiella ictaluri* exoantigen was significantly higher in channel catfish fed diets supplemented with either 30 or 300mg kg body $weight^{-1}$ iron methionine for 14 weeks. However, these dietary inclusion levels failed to influence survival of juvenile channel catfish against *E. ictaluri* 14-days post challenge (Lim *et al.* 2000). Rumsey *et al.* (1994) demonstrated that trout fed soybean protein exhibited enhanced leucocyte phagocytic activity, bacterial killing and superoxide production. Krogdhal *et al.* (2000) found that the inflammatory response caused by soybean inclusion in feed correlated with decreased resistance to *A. salmonicida*. Finally, carotenoids such as beta-carotene have been shown to elevate both humoral (Amar *et al.* 2000) and cellular (Amar *et al.* 2001) factors in trout.

## Conclusion

There is a diverse range of products that have been tested for their immunostimulatory properties in fish. Such products are classified as either killed pathogens and their products, β-glucans, chitin, unidentified animal extracts, synthetic chemicals, plant molecules, nutrients, RNA or host/animal molecules. However, relatively few of the products that show promise in a research context become available for use by the fish farmer. Legislative considerations, efficacy of the product in the field and economic factors are some of the common barriers to commercialisation of novel immunostimulants.

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SCIENTIFIC PAPER

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# Sampling errors in examining salmon (*Salmo salar* L.) for sea lice (*Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* Nordmann) burden: a comparison of methods

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*Abstract*

*Two methods of examining Atlantic salmon,* Salmo salar*, for the sea lice* Lepeophtheirus salmonis *and* Caligus elongatus*, were compared. The results obtained show that anaesthetising the salmon and examining them at the farm site gives statistically significantly higher sea lice infestation rates than if fish are killed by a blow to the head. It was established that the same fish examined on-site after being anaesthetised lose significant numbers of sea lice (approximately 25%) upon re-examination in the laboratory. Analyses of* L. salmonis *distribution on the fish surface showed that there appears to be a preference for site location on the fish body by* L. salmonis*. Mobile stages were found mainly in the posterior dorsal area of the body above the lateral line from the posterior end of the dorsal fin to the caudal fin with numbers of attached stages being highest on the ventral fins.*

## Introduction

Sea lice infestations are frequently found on Irish fish farms and are controlled using a range of different methods, including management techniques and chemotherapeutic treatments. Two species of sea lice occur on Atlantic salmon, *Salmo salar* L., in Irish marine waters, *Lepeophtheirus salmonis*

(Krøyer) and *Caligus elongatus* Nordmann. The former parasitises salmon and other salmonids and the latter is found on over eighty different species of marine fish including salmonids. *L. salmonis* has the more serious impact of these two species, occurring more frequently on farmed salmon (Jackson and Minchin, 1993). Descriptions of the life cycle for *L. salmonis* are given in Kabata (1979) and Schram (1993), and for *C. elongatus* by Hogans and Trudeau (1989) and Piasecki (1996).

The purpose of the study undertaken was to investigate sources of error in sampling procedures of sea lice infestations. There are a number of ways in which errors can be introduced in such investigations: fish capture method, immobilisation techniques, handling methods and storage of the fish sample before examination, counting and identifying sea lice stages. Weather conditions can also be a cause of concern in terms of visibility, with bad weather having a negative effect on the numbers of sea lice seen.

It is known that capture method affects the levels of sea lice recorded in salmonid fish (Nagasawa, 1985; Holst *et al*., 1993; Tully *et al*., 1999). In the present study salmon were captured following the method recommended by Jackson *et al*. (1997). It is highly likely that losses of sea lice numbers result from the type of capture method used, however, the present study seeks to investigate the levels of errors introduced when immobilising and handling the fish sample following capture, and not due to capture method. Sea lice numbers were estimated following either anaesthetisation followed by examination on-site or killing by a blow to the head and laboratory examination. The former were also re-examined on return to the laboratory.

The distribution of the different sea lice stages on the fish body was also examined to determine if specific sites lose different stages of sea lice more readily than others during examination, and also to determine if sites are favoured by specific stages of sea lice. The purpose of the present study was to determine a methodology for later studies, where the fish would not be sacrificed before analyses. It was therefore decided not to examine the gill cavity for sea lice, which would place unnecessary stress on the fish.

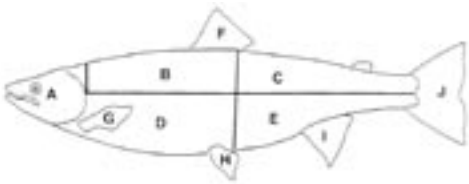
## Materials and Methods

Both *L. salmonis* and *C. elongatus* were sampled during the study, with stages visible by the naked eye down to copepodid level being counted. However, distinguishing between the two species at this level, and at the first

chalimus stage, would not be possible unless examined by a microscope. It was decided therefore, for the purpose of the study, that these stages would be combined for the two species of sea lice counted on-site at sea. The operators undertaking the sampling were experienced in sea lice infestation investigations and carried out all the counting in the study. The samples of fish were collected at a salmon farm in Bertraghboy Bay on the west of Ireland. Fish were attracted to the surface of the water with food and then caught using a coarse meshed hand-net. Two samples of twenty-three and twenty-five fish respectively were sampled on the 13th March 2003. These fish were divided into fish killed by anaesthesia and those killed by a blow to the head.

The fish killed by anaesthetic were placed into individual buckets containing a lethal dose of Tricaine methane sulphonate (MS222). They were then examined individually and length, weight, sea lice numbers and sea lice distribution on the body were noted. To examine sea lice distribution the method used by Bjørn and Finstad (1998) was employed. This method examines the distribution of sea lice on the surface of the fish using different zones on the body (Figure 1). The water from the buckets containing the anaesthetic was filtered through fine mesh after each fish was examined to check the levels and stages of sea lice that were detached during the sampling process. Any sea lice found at this point were placed into a separate bottle and labelled as a bin bottle for that fish. The fish were then placed into individual plastic bags and cooled for transportation to the laboratory.

FIG 1: Areas of distribution analysed for sea lice during the study (after Bjørn and Finstad, 1998).




**Locations:**

A head and operculum
B anterior dorsal area of the body above the lateral line from the head to the posterior end of the dorsal fin
C posterior dorsal area of the body above the lateral line from the posterior end of the dorsal fin to the caudal fin
D anterior ventral area of the body below the lateral line from the head to the posterior end of the dorsal fin
E posterior ventral area of the body below the lateral line from the posterior end of the dorsal fin to the caudal fin
F dorsal fin
G pectoral fin
H ventral fin
I anal fin
J caudal fin

Fish killed by a blow to the head were first placed into individual plastic bags to minimise the loss of sea lice, then killed, cooled and transported to the laboratory. There, the fish were examined for length, weight, sea lice counts and sea lice distribution. In the laboratory fish previously examined on-site after anaesthetisation were re-examined as before. The individual plastic bags containing both samples of fish were also examined for any detached sea lice.

A further sample of fifty-nine salmon was taken on the 25th March from the same cage as before. All of these fish were killed by a lethal dose of MS222 and examined as for the anaesthetised fish above.

Kruskal-Wallis analyses were performed to test for differences in lengths and weights in the fish samples. Mann Whitney U tests were used to test various pairs of data relating to sea lice counts. Chi square tests ($\chi^2$) were also used and where two categories were investigated the Yates correction was employed. Results were considered significant at 95% confidence limits. Wilcoxon signed rank tests were used to test data relating to paired samples.

## Results

Details of the samples of salmon examined during the course of the study are given below in Table 1. The relationships between fish length and weight for each of the samples were $y = 4.4883x - 958.8$ (sample 1), $y = 3.9537x - 809.43$ (sample 2) and $y = 4.355x - 920.93$ (sample 3) (y = weight (g), x = length (mm)). No significant differences were observed between the lengths and weights for the three samples of fish examined (Kruskal-Wallis, $p > 0.24$ for both length and weight).

TABLE 1: Sample details of the fish examined during the study, with mean lengths and weights ± standard deviations (SD).

| Date | Details of sample | Sample no. (n) | Mean length (mm) ± SD | Mean weight (g) ±SD |
|---|---|---|---|---|
| 13/03/03 Sample 1 | Killed by anaesthesia | 23 | 322.78±23.36 | 396.26±101.69 |
| 13/03/03 Sample 2 | Killed by blow to head | 25 | 319.60±21.92 | 374.42±87.82 |
| 25/03/03 Sample 3 | Killed by anaesthesia | 59 | 328.61±30.84 | 422.92±128.81 |

A summary of sea lice counted for each sample of fish during the various handling procedures in the experiment are shown in Table 2. There were no significant differences in the sea lice numbers recorded following anaesthetisation on the two dates sampled (samples 1 and 3), either on-site or in the laboratory ($p = 0.932$ (on-site), $p = 0.804$ (lab)), Mann Whitney U test). When the sea lice counts from these samples were pooled and compared to the counts from sample 2, the blow to the head killed fish, a significant difference was noted ($p < 0.0001$, $U = 1566.5$) (Mann Whitney U tests).

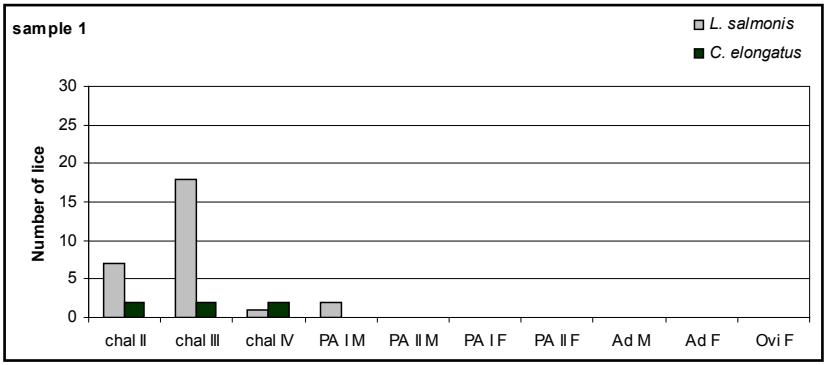
TABLE 2: Summary of sea lice counts for each of the samples examined on-site and on return to the laboratory. Mean sea lice counts (SL) per sample are given ± standard deviations (SD). Sea lice counts recovered from the bags and bins are given separately and were not included in overall counts.

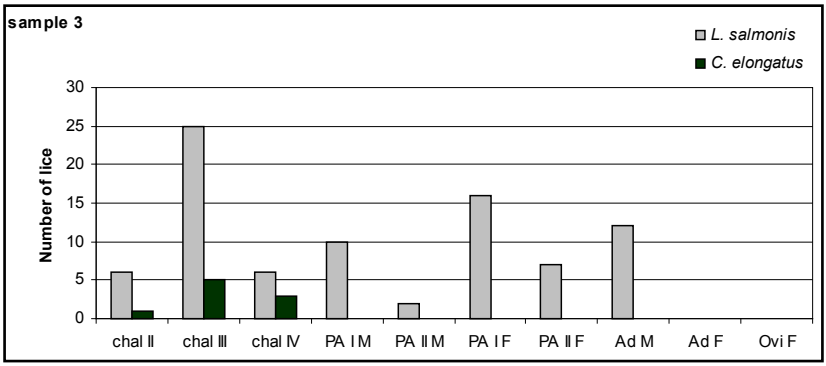
| Sample | On-site sea lice counts | Sea lice lab counts | % lost |
|---|---|---|---|
| Sample 1 | 40 (5 in bin) | 30 (4 in bag) | 25% |
| Mean SL±SD | 1.74±1.79 | 1.30±1.77 | |
| Sample 2 | - | 10 (2 in bag) | - |
| Mean SL±SD | | 0.42±0.58 | |
| Sample 3 | 101 (28 in bin) | 75 (3 in bag) | 25.7% |
| Mean SL±SD | 1.71±1.64 | 1.27±1.46 | |

Analyses of sea lice counts from anaesthetised fish only, which were subsequently transported back to the lab and again examined, showed that a relatively high percentage (25%) of the sea lice counted attached to the fish on-site were absent on reanalyses in the laboratory (Table 2). This was despite inclusion of any loose sea lice found in the bins and plastic bags in which the fish were contained. The difference between the counts on-site, including bin numbers, and those in the laboratory, including those found loose in the bags, was statistically significant ($T = 153.00$, $p = 0.035$ (sample 1) and $T = 1392.00$, $p < 0.001$ (sample 3), Wilcoxon signed rank tests).

Frequency distributions of the different life stages of *L. salmonis* and *C. elongatus* are shown in Figure 2 for the anaesthetised fish in samples 1

FIG 2: Frequency distributions of the different sea lice life cycle stages in samples 1 and 3 following initial counts.




and 3. Small numbers of sea lice were recorded from sample 2 and so little can be said about sea lice distributions. Also, sample 3 showed later preadult and adult male stages than in sample 1, a result expected as this sample was examined twelve days later than the first, by which time sea lice had developed further.

Because of the very low numbers of *C. elongatus* recorded during the study (n = 27 for all samples combined) only the distribution of *L. salmonis* on the surface of the salmon was examined (Table 3). The copepodid and chalimus I stages which were not identified to species level were, however, examined. No chalimus I, adult female or ovigerous female *L. salmonis* were recorded from any of the fish examined. In sample 1 mainly attached stages were

TABLE 3: Percentage frequency distribution of the copepodid stage of both *L. salmonis* and *C. elongatus* and the following developmental stages of *L. salmonis* for each zone of the fish examined.

| **sample 3 (n = 59)** | | **Zone** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Stage** | **n** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **Bin** |
| **Copepodid (both sp.)** | 8 | 0 | 0 | 0 | 12.5 | 0 | 0 | 0 | 87.5 | 0 | 0 | 0 |
| *L. salmonis* | | | | | | | | | | | | |
| Chal II | 6 | 0 | 0 | 50.0 | 33.3 | 16.7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chal III | 26 | 0 | 0 | 11.5 | 19.2 | 15.4 | 19.2 | 3.8 | 15.4 | 0 | 11.5 | 3.8 |
| Chal IV | 6 | 0 | 0 | 0 | 16.7 | 16.7 | 16.7 | 16.7 | 16.7 | 0 | 16.7 | 0 |
| PA I M | 14 | 21.4 | 0 | 42.9 | 7.1 | 0 | 0 | 0 | 0 | 0 | 0 | 28.6 |
| PA II M | 7 | 0 | 0 | 14.3 | 0 | 14.3 | 0 | 0 | 0 | 0 | 0 | 71.4 |
| PA I F | 24 | 4.2 | 12.5 | 33.3 | 8.3 | 0 | 0 | 0 | 0 | 8.3 | 0 | 33.3 |
| PA II F | 8 | 12.5 | 0 | 62.5 | 0 | 12.5 | 0 | 0 | 0 | 0 | 0 | 12.5 |
| Ad M | 13 | 15.4 | 15.4 | 53.8 | 0 | 7.7 | 0 | 0 | 0 | 0 | 0 | 7.7 |

Chal = chalimus, PA = pre-adult, Ad = Adult, Ovi = ovigerous, M = male, F = female

recorded, with only 3 mobile pre-adult I males being found. No clear preference for different regions of the fish could be discerned for the attached stages. In sample 2 no mobile stages were recorded, 12 attached stages were counted (4 of which were copepodids) and again no clear preferences could be identified because of the low numbers. The larger sample 3 perhaps gives a better understanding of these preferences, as larger numbers of later stages were present. Juvenile mobile *L. salmonis* stages appear to concentrate on zone C in this sample. Adult males were also found to be more frequent on zone C. Chi square tests on the results show that when the body zones are combined and the fin zones are combined there was no significant difference between these two areas for attached *L. salmonis* stages ($p > 0.5$, $\chi^2 = 0.270$ df = 1), however there was a significant difference for mobile *L. salmonis* stages ($p < 0.001$, $\chi^2 = 39.362$, df = 1). Looking at the zones individually (A-J) a significant difference was observed for both attached and mobile *L. salmonis* stages ($p = 0.01$, $\chi^2 = 21.649$, df = 9 for attached: $p < 0.001$, $\chi^2 = 128.532$, df = 9 for mobile). Highest numbers of attached stages were found on zone H and for mobile stages on zone C.

As expected, a greater proportion of mobile stages tended to have lost contact with the host after transportation to the laboratory for re-examination ($\chi^2 = 5.093$, $p < 0.025$, df = 1); 19 mobile compared to 9 attached were lost into the bin (Table 4). A relatively high number of mobile stages (n = 6) were also lost from zone C upon re-examination in the laboratory, although this loss

TABLE 4: Details of the distribution of attached and mobile sea lice on each of the zones examined on-site and in the laboratory for sample 3, together with percentages lost after re-examination in the laboratory.

| Zones | On-site sea lice counts | | Sea lice lab counts | | % lost after lab counts | |
|---|---|---|---|---|---|---|
| | Attached | Mobile | Attached | Mobile | Attached | Mobile |
| **A** | 1 | 7 | 0 | 5 | 100.00 | 28.60 |
| **B** | 0 | 5 | | 3 | | 40.00 |
| **C** | 7 | 27 | 3 | 21 | 57.10 | 22.20 |
| **D** | 11 | 3 | 7 | 3 | 36.40 | 0.00 |
| **E** | 6 | 3 | 5 | 2 | 16.70 | 33.30 |
| **F** | 7 | 0 | 7 | | 0.00 | |
| **G** | 2 | 0 | 1 | | 50.00 | |
| **H** | 14 | 0 | 12 | | 14.30 | |
| **I** | 1 | 2 | 0 | 0 | 100.00 | 100.00 |
| **J** | 5 | 0 | 3 | | 40.00 | |
| **Bin** | 9 | 19 | | | | |
| **Bag** | | | | 3 | | |

was not statistically significant. The observed ratio between attached versus mobile numbers of sea lice found on-site were shown to be similar to those recorded in the laboratory ($\chi^2 = 0.026$, $p > 0.75$, $df = 1$).

## Discussion

The decision to examine the methods used for sea lice analyses on salmon was undertaken because further studies to be undertaken by the authors required a proven method to determine accurate levels of sea lice and their stages on salmon, without having to sacrifice the fish. Significant differences were recorded in the sea lice counts between fish treated differently at examination. This has profound implications for comparisons on sea lice burdens within and between studies. The fish examined in each of the three samples were not significantly different in size, and size, therefore, can be rejected as an influence on differences in the sea lice numbers observed. Significantly lower numbers of sea lice were recorded from the fish killed by a blow to the head, the sea lice presumably having been lost as a result of greater handling before killing. This is consistent with the results of Jackson and Minchin (1993) who found lower numbers of mobile sea lice stages on fish that were killed by a blow to the head compared to those that had been anaesthetised.

However, their differences were not statistically significant. In the present study, for practical purposes on the sea cage, the first 23 fish were anaesthetised (sample 1) and the second 25 were killed with a blow to the head (sample 2). Thus, it is possible that the earlier fish caught (sample 1) might have been more responsive to food than the second batch of fish caught (sample 2) and had a higher probability of capture. Should responsiveness to food be linked to sea lice burden, then the results of this study would be confounded. To test the hypothesis that earlier caught fish had a different sea lice burden to fish caught later, the sea lice burden from the first 30 fish caught on the second day of sampling (sample 3, $n = 59$) were tested against the sea lice burden from the last 29 fish caught. No significant difference in sea lice burden was evident ($U = 426.5$, $p = 0.895$).

Significant numbers of sea lice were lost from fish anaesthetised on-site and examined later in the laboratory ($p = 0.035$ (sample 1) and $p < 0.001$ (sample 3)). This suggests that sea lice numbers may be seriously underestimated in studies where fish are examined in the laboratory, having been transported there following sampling on-site. However, it should be noted that a second handling step, a field examination, was included in the present study.

There does appear to be a preference for site location on the fish body by *L. salmonis* (Table 3). Mobile stages were found to prefer zone C, the area behind the dorsal fin, above the lateral line, to the tail. Both juvenile and adult male *L. salmonis* were found to be more concentrated on this zone than any other, with Chi square tests showing that a preference for different zones was evident.

Various combinations of the methods for immobilisation employed during the present study have been used for analyses of sea lice from a range of fish species including Atlantic, Chinook and Coho salmon, rainbow trout and sea trout (Bjørn and Finstad, 1998; Bruno and Raynard, 1994; Dawson, 1998; Finstad *et al*., 2000; Grimnes and Jakobsen, 1996; Jackson and Minchin, 1993; Johnson and Albright, 1992; Mackenzie *et al*., 1998; Mo and Heuch, 1998; Mustafa *et al.*, 2000; Ruane *et al*., 2000; Tully *et al*., 1999). From the results of this study, the most accurate method the authors found for examining salmon for sea lice numbers following capture was by examining the fish on-site after anaesthetising them. As the fish do not need to be sacrificed, large numbers of fish can be examined without adverse effects. Minimum losses of sea lice into the bin at this point can be observed, when compared to the sea lice losses seen when killing fish by a blow to the head and then examining them in the laboratory.

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SCIENTIFIC PAPER

*Fish Veterinary Journal (2005) (8) 45–61*

# Minimum inhibitory concentrations to selected antibiotics of motile aeromonads isolated from ornamental fish shipments from Singapore and South America into the UK

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*Abstract*

*Minimum Inhibitory concentrations (MIC) of motile aeromonads isolated from ornamental fish shipments into the UK from Singapore and South America against 3 antibiotics currently licensed for use in aquaculture in the UK and enrofloxacin were determined by agar doubling dilution method. The MIC of a high percentage of isolates from Singapore fell into the category of resistant when tested against oxytetracycline, potentiated sulphonamide and oxolinic acid. Most aeromonads from South America showed high MIC values for oxytetracycline, but were mildly susceptible to potentiated sulphonamide and completely susceptible to oxolinic acid and enrofloxacin. Enrofloxacin MIC for the Singaporean strains were variable, as isolates had a tendency to spread over the range tested for. Considering that most Singaporean aeromonad isolates showed resistance to Oxolinic Acid, results for the new 4-quinolone suggests that enrofloxacin-resistant opportunist bacterial pathogens are emerging in the ornamental fish trade from Southeast Asian sources.*

## Introduction

The quantity of antibiotic used when treating a large number of animals is based on striking a balance between economics and the efficacy of the

treatment. The aim is to achieve the maximum therapeutic effect on the subject animals treated, whilst avoiding overdosing which can either be harmful to the subject or wasteful in terms of the quantity of antibiotic used. Antimicrobials are used in large-scale production of tropical ornamental fish, mostly in a preventive fashion. Commonly, the treatment is carried out by delivering the antibiotic into the holding water (Meinelt and Pietrock 2001). Assessment of the efficacy of antibacterials when applied by the former method to treat tropical ornamental fish is very subjective, since clinical concentrations in internal organs are not normally reported or parallel *in vitro* test carried out (Reimlinger *et al.* 1990). Control of the outbreak is indicated by recovery of the treated subjects (Alcaide *et al.* 2001).

In the field of veterinary therapeutics, the clinical efficacy of any antibacterial drug can be assessed *in vitro* by determining the bacterial susceptibility to the particular drug used. Methods used to determine bacterial susceptibility *in vitro* can be divided into methods carried out on agar or methods carried out in broth (turbidimetric methods) (Lorian 1986). In aquatic animals achieving four times the *in vitro* Minimum Inhibitory Concentration (MIC) in the target tissues has been suggested as optimal for some antibacterial drugs used to treat bacterial fish disease (Tsoumas, Alderman and Rodgers 1989). MIC is defined as "the lowest concentration that completely inhibits bacterial growth, disregarding a single colony or a faint haze caused by inoculum" (Prescott and Baggot 1985).

In a previous report linked to the present paper, motile aeromonad isolates obtained from ornamental fish shipments into the UK from Singapore and South America, were tested for their resistance to 11 antibiotics by the Kirby-Bauer disc diffusion method. Antibiotic multiresistant bacteria from both these places of origin were discovered in broadly similar proportions, although some bacteria responded differently to specific antibiotics, such as oxolinic acid, apramycin and enrofloxacin (del Rio-Rodriguez and Turnbull 2002).

This paper is the final one in a series of papers arising from a larger study on ornamental tropical fish imported into Scotland. The last part of the work was aimed at determining the *in vitro* MIC of 3 antimicrobials authorised for use in aquaculture fish in the UK (potentiated sulphonamide, oxolinic acid and oxytetracycline) and also enrofloxacin against bacterial isolates previously recovered between 1996–1998.

## Materials and methods

The minimum inhibitory concentration for test and control bacteria followed the agar doubling dilution technique (Lorian 1986). The materials and methods for this technique were adapted from the Standard Operation Procedures (SOP/009, 010, 023, 024, 025, 026, 039 and 040) used in the bacteriology laboratory of the Institute of Aquaculture, University of Stirling.

**Selection of test and control bacteria**

The MICs for enrofloxacin (ENRO), oxolinic acid (OA), oxytetracycline (OT) and potentiated sulphonamide (SXT) were determined for 46 isolates of motile *Aeromonas* spp. from Singapore and 12 from South America. Three other bacterial isolates donated from the bacterial collection at the bacteriology laboratory, Institute of Aquaculture, University of Stirling were used as controls on the accuracy of the tests. These isolates were chosen because their MICs for the antibiotics tested were previously established at the above laboratory (Table 1). These isolates with pre-determined MICs are henceforward referred to as control isolates.

TABLE 1: MIC of control bacteria isolates obtained in previous work

| CRTLS | OT | OA | SXT | ENRO |
|---|---|---|---|---|
| *Aeromonas salmonicida*[1] 1102 | 0.016 | 0.04 | 0.6 | <0.00.5 |
| *Escherichia coli* [2] 10418 | 0.6 | 0.16 | 0.6 | -* |
| *Aer. salmonicida*[3] FCS | - | - | - | <0.005 |

*MIC laboratory data for enrofloxacin was not available for *E. coli* NCTC 10418. *A. salmonicida* FCS was used instead.

[1]National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland

[2]National Collection of Type Culture (Public Health Laboratory Services, London, England)

[3]Isolated in a furunculosis outbreak in a private salmon farm in Scotland, pathogenic strain (D. Cox, Marine Harvest, Scotland).

The control bacteria from the Institute of Aquaculture were obtained from lyophilized isolates and resuscitated in tryptone soy broth (TSB) incubated at 22˚C for 24 hours. Once the isolates were resuscitated they were maintained on tryptone soy agar (TSA) plates incubated at 22˚C. Sub-culturing of each control isolate was carried out every other day to maintain Log-phase growth during the period of the study.

The tests isolates were maintained on TSA slants. These were checked for purity on TSA plates and maintained by serial subculture during the MIC

TABLE 2: Antibiotics and the method used in order to obtain the intended concentration range

| Antibiotic | Procedure to get a stock solution of 160 μg/ml |
|---|---|
| Oxolinic acid | 0.008 g of OA dissolved into a 5% NaOH 1M, freshly made and then diluted with sterile distilled water (SDW) up to 50 ml. |
| Oxytetracycline hydrochloride | 0.0087 g dissolved in 50 ml of SDW. |
| Potentiated sulphonamide (Borgal 240 mg/ml) | 1 ml of Borgal was diluted in 9 ml SDW to produce 24 mg/ml. 1 ml of this solution was added to 2 ml of SDW to produce 8 mg/ml. 1 ml of the latter solution was added to 49 ml of SDW to produce 160 μg/ml |
| Enrofloxacin (Baytril 100 mg/ml) | 1 ml of Baytril was added to 9 ml of Sterile distilled water (SDW) to produce a concentration of 0.010 g/ml. Two ml were extracted from this solution and the 8 ml left were then added to 42 ml of SDW achieving the desired concentration of 160 μg/ml. |

tests. Sub-culturing of each test isolate was also carried out every second day to maintain Log-phase growth.

**Preparation of test and control bacteria working inocula for MIC**

Two to three bacterial colonies of each species were removed from the TSA plates with a sterile bacteriology loop and inoculated into 5 ml of TSB contained in sterile universal bottles and stored at 4°C overnight. The following day, they were removed from the 4°C, inverted gently and transferred to 22°C where they were kept for 2 to 3 hours in order to achieve exponential growth (TSB-Log). The working inoculum was then obtained by transferring 0.5 ml of TSB-Log into 4.5 ml iso-sensitest broth (SB) within sterile universals. Ten out of the 60 working inocula were randomly chosen on each occasion for standard bacterial counts on TSA. The mean CFU (number of colony forming units) of the working inocula was $1.5 \times 10^5$ per ml but ranged from of $1.0 \times 10^4$ to $3.2 \times 10^5$ CFU/ml.

**Antibiotic stock solutions and standards**

The antimicrobials tested and their producers were OA, OT (Sigma Aldrich

UK), ENRO (Baytril®, Bayer Germany) and SXT (Borgal®, Hoechst, UK, Ltd.).

Stock solutions were prepared following the procedures described in Table 2. Each antibiotic was placed into 50 ml of sterile distilled water within a 50 ml volumetric flask to give a concentration of 160 μg/ml (stock solution). The standards were prepared in duplicate, by double-diluted serial transfer, with 10 ml of the stock solution into pre-labelled universal bottles containing 10 ml of distilled water.

The standard 80μg/ml concentration was prepared by transferring 10 ml of the stock to an empty universal bottle. The rest of the concentrations were from 40 down to 0.005 μg/ml. The final concentrations were achieved when the solutions were mixed with the agar. Care was taken in shaking the bottles to mix the antibiotic and when removing the 10 ml for the next concentration.

**Preparation of MIC plates**

Iso-sensitest agar® (ISO) (Unipath Ltd) was prepared by adding 25.12 g into 400 ml of distilled water and autoclaving at a pressure of 120 psi for 20 minutes. After autoclaving the bottles containing the agar were transferred to water baths (50 ºC) in order to keep the media liquid.

With the aid of sterile pipettes 10 ml of agar was added to the universals containing the standard antibiotic solutions (from lower to higher concentration), mixed by gently inverting the universals and then poured onto Petri dishes previously labelled with the corresponding concentration.

Each plate received a volume of 20 ml. Care was taken to evenly distribute the agar on the plate. The calculated concentrations achieved for the agar plates were then 80, 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.16, 0.08, 0.04, 0.02, 0.01 and 0.005 μg/ml. Plates were made in duplicate and used the same day.

**Plates inoculation and interpretation.**

A multi-point inoculator machine (Herga Electric Ltd. Suffolk, England) was used for the inoculation of the doubling agar dilution plates. The multi-pointer, the holder and the template were previously sterilised by autoclaving.

From the inoculated TSB log phase, 0.5 ml were transferred to previously prepared and labelled universals containing 4.5 ml of TSB and mixed. With the aid of a Gilson pipette with exchangeable sterilised tips, 0.25 ml of the working inocula were added to assigned wells of the template (Figure 1).

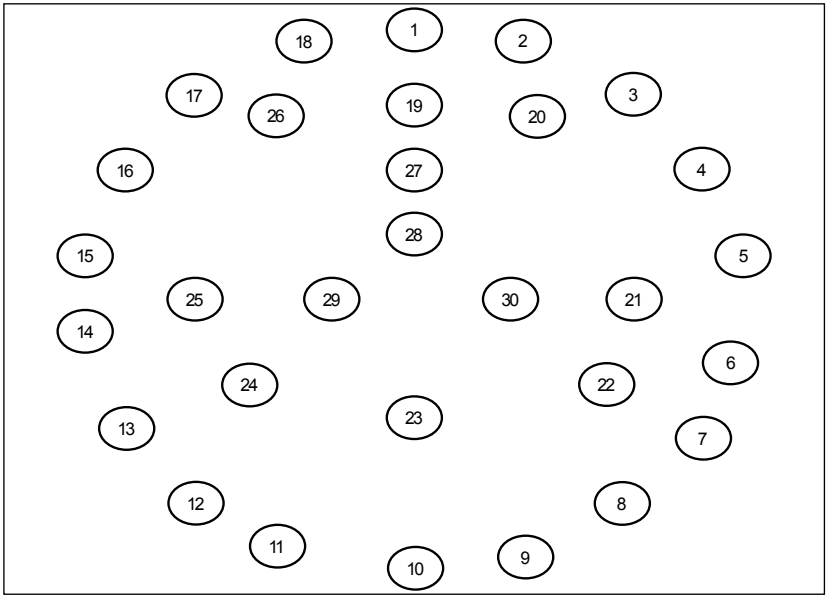


FIG 1: Numbers on this figure representing the wells template correspond to the numbers assigned to the isolates in Table 3

The template was put into position according to the manufacturer specifications for the multi-point inoculation machine. Bacteria were placed into the template according to their allocated number (Table 3). The plates were then inoculated from lower to higher concentration and a control growth plate with no antibiotic was inoculated at the beginning and another at the end of a series. The pins of the inoculation device were flamed between duplicates.

Inoculated plates were left to dry for 30 minutes before being inverted and incubated at 22°C and checked for growth at 24 and 48 h. MICs were recorded at 24 and rechecked at 48 hours, as *A. salmonicida* grows slower than motile aeromonads. MIC was recorded following the definition of Prescott and Baggot (1985). The highest MIC was recorded in the cases where the MIC of a strain did not produce identical results in both replicates.

## Results

### Minimum Inhibitory Concentrations for control and test bacteria

MIC for OA obtained in this study for *A. salmonicida* 1102 and *E. coli* 10418 (Table 4) differed from the results obtained in previous laboratory tests, but

TABLE 3: Aeromonads isolates**

| Batch A | | Batch B | |
|---|---|---|---|
| 1. 2V22BFPO | 16. 1VI21SFPO | 1. F4-2 PLATY-CREAM | 16. 1V2 SFPO |
| 2. 1VI21BFPO | 17. 1III1BFPO | 2. TANK 5 TSA | 17. F9-1 PLATY |
| 3. 1II10BFPO | 18. F59-2CREAM | 3. F8-1 PLATY-CREAM | 18. 1I TESATSA |
| 4. 2IV11BFPO | 19. F60-1CREAM | 4. TK5 4-1 CREAM | 19. 1IHTSATSA |
| 5. 2V11BFPO | 20. F65-CREAM | 5. F3-3 WITHISH | 20. 2IV MELASA |
| 6. 1V13BFPO | 21. F61CREAM | 6. F6 CREAM | 21. SFSACLBF1 |
| 7. 1IV5BFPO | 22. F58CREAM | 7. F10 CREAM | 22. 1II1SFSA |
| 8. 1VI18BFPO | 23. F70-1CREAM | 8. F14 CREAM | 23. 1II2 BFSA |
| 9. 2IV1BFPO | 24. F63-1CREAM | 9. F16-1 CREAM | 24. 2VI1 BFSA |
| 10. 1VI11SFPO | 25. F28-3C/Y | 10. F18 CREAM | 25. 2III17BFSA |
| 11. 3II3SFPO | 26. F28-1CREAM | 11. F20 CREAM | 26. F16-2 PALE |
| 12. 1III1SFPO | 27. F28-1PALE | 12. F27-2 PALE | 27. 1I19 BFSA |
| 13. 4V16SFPO | 28. F28-2PALE | 13. 1II SWRPO | 28. 1III1 BFSA |
| 14. 1III13SFPO | 29. *E. coli* NCTC 10418* | 14. 4V TIGERCI | 29. 2II25 BFSA |
| 15. F27-1CREAM | 30. *A. salnomicida* 1102* | 15. 2 IV PLATY WB | 30. 2I2 BFSA |

*Control isolates. *E. coli* NCTC 10418 was replaced by *A. salmonicida* FCS, Bacteriology Lab, Institute of Aquaculture collection for enrofloxacin.
**Isolates codes only of internal importance. South American isolates in grey

this difference was only a one-fold dilution. The MIC for OT, SXT and ENRO coincided with previous laboratory results.

With respect to the test bacteria, in general motile aeromonads from Singapore and South America produced variable results to all of the antibi-

TABLE 4: Previously determined MIC (μg/ml) of control bacteria and MIC obtained in this work

| Controls | OT | OA | SXT | ENRO |
|---|---|---|---|---|
| *A. salmonicida* 1102 | 0.016<br>(0.016) | 0.02<br>(0.04) | 0.6<br>(0.6) | <0.00.5<br>(<0.005) |
| *E. coli* 10418 | 0.6<br>(0.6) | 0.08<br>(0.16) | 0.6<br>(0.6) | -* |
| *A. salmonicida* FCS | - | - | - | <0.005<br>(<0.005) |

*Control isolates. *E. coli* NCTC 10418 was replaced by *A. salmonicida* FCS, Bacteriology Lab, Institute of Aquaculture collection for enrofloxacin.

otics tested. However, a trend was observed for the strains from Singapore which appeared to have MIC values higher than those obtained from the South American aeromonads (Table 5).

**Oxytetracycline hydrochloride**

For the Singaporean isolates the lowest MIC for OT was 0.08 µg/ml, however, up to 12 isolates had a MIC higher than 80 µg/ml (Figure 2). Aeromonads' MIC distribution was very similar for both origins. However, for the South American strains the lowest MIC recorded was 0.16 µg/ml, and one of the Singaporean isolates had a MIC lower than 0.16 µg/m (Figure 2). Most of the Isolates from both places of origin had MICs near the upper range tested for in this study (10 to >80 µg/ml).

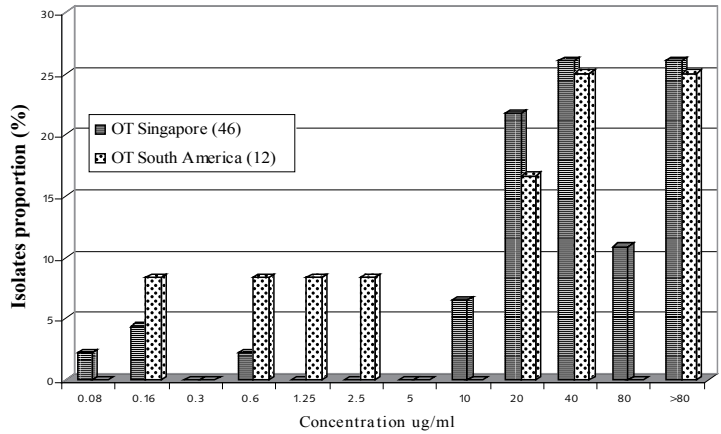


FIG 2: MIC isolates proportion distribution for OT

***Oxolinic acid (OA)***

Thirty aeromonad bacterial isolates from Singapore had an OA MIC higher than 80 µg/ml (the highest concentration tested for in this study) (Figure 3). The other 16 Singaporean isolates had a MIC for OA which spread over a range of concentrations from 1.25 to 80 µg/ml. In contrast, no aeromonad isolates from South America showed a MIC greater than 10 µg/ml, and the MIC of these isolates for OA ranged from <0.005 to 5 µg/ml (Figure 3).

***Potentiated sulphonamide (SXT)***

Twenty-one aeromonad isolates from Singapore had MICs for SXT higher

TABLE 5: MIC Frequency of motile aeromonad isolates

| Antimicrobial | Minimum Inhibitory Concentration of antibacterial agent µg/ml | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates origin** | **≤0.005** | **0.005** | **0.01** | **0.02** | **0.04** | **0.08** | **0.16** | **0.3** | **0.6** | **1.25** | **2.5** | **5** | **10** | **20** | **40** | **80** | **>80** |
| OA Singapore | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 3 | 4 | 1 | 0 | 2 | 30 |
| OA South Am. | 0 | 0 | 0 | 3 | 3 | 2 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| OT Singapore | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 0 | 0 | 3 | 10 | 12 | 5 | 16 |
| OT South Am. | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 2 | 3 | 0 | 3 |
| SXT Sing | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 7 | 7 | 4 | 2 | 3 | 1 | 0 | 21 |
| SXT South Am | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 4 |
| ENRO Sing | 2 | 0 | 2 | 1 | 4 | 8 | 15 | 7 | 3 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ENRO South Am | 10 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

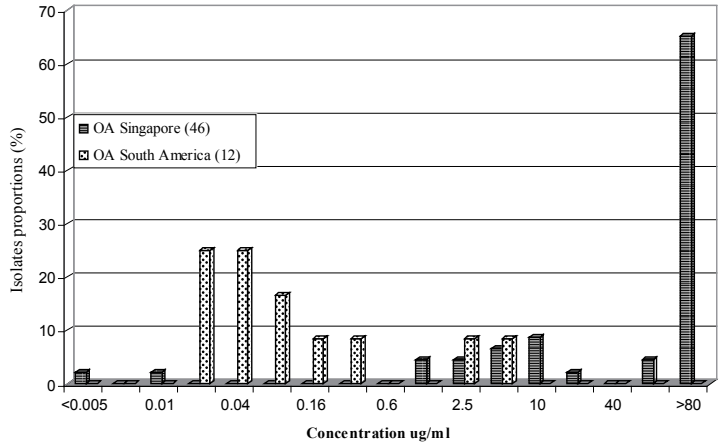


FIG 3: MIC isolates proportion distribution for OA

than 80 µg/ml. However, the MICs of 24 of these isolates were near the lower range of concentrations tested. In contrast, only 4 isolates from South America had a MIC for SXT higher than 80 µg/ml.

The MICs of South American isolates for SXT were spread over a range from 0.6 to 40 µg/ml. The lowest MIC for aeromonads from both places of origin was 0.6 µg/ml (Figure 4).

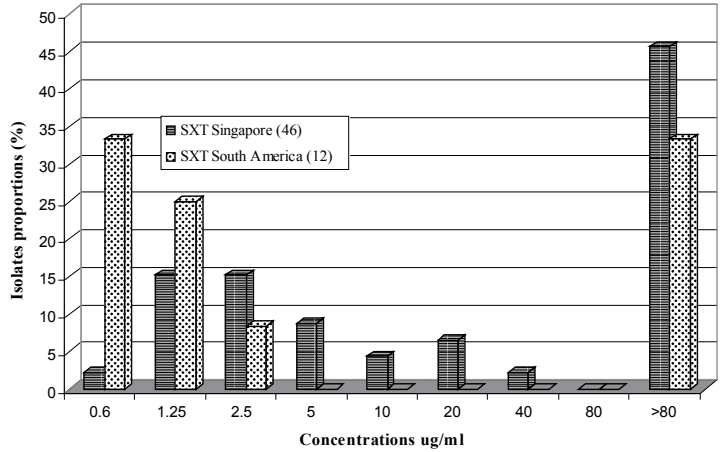


FIG 4: MIC isolates proportion distribution for SXT

***Enrofloxacin (ENRO)***

The most marked contrast in response between Singapore and South America aeromonad isolates was observed in the MICs for ENRO (Figure 5).

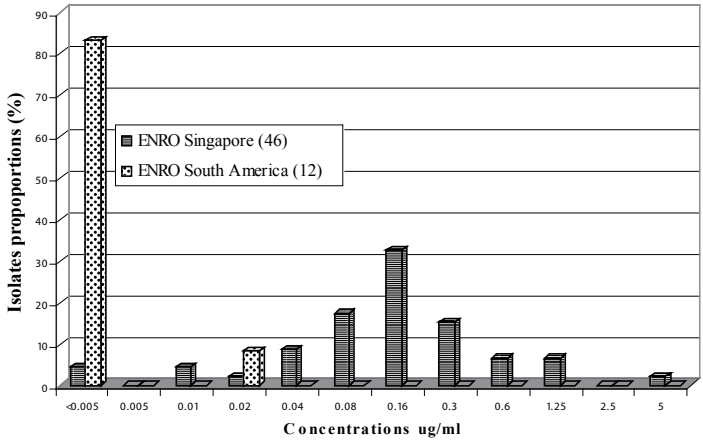


FIG 5: MIC isolates proportion distribution for ENRO

While the MIC of the Singaporean isolates ranged between 0.04 to 0.3 μg/ml, almost all isolates from South America had a MIC lower than 0.005 μg/ml. The highest MIC recorded in this work was for isolate "8" (5 μg/ml) batch A from Singapore, but most of these isolates appeared to have a MIC at 0.16 μg/ml.

Control bacteria had a MIC below 0.005 μg/ml which was consistent with previous laboratory work on these bacteria by the bacteriology laboratory of the Institute of Aquaculture.

***Discussion***

During the period in which this work was carried out, interpretative standards for the susceptibility of bacterial pathogens to OA and ENRO could not be found in the literature and manufacturers were not able to provide this information. The National Committee for Clinical Laboratory Standards (NCCLS, USA 1992) has established standards for OT and SXT, and although quinolones are also included, OA and ENRO are not, probably because they are used only in veterinary medicine. Significant efforts are being made to develop methodologies of standard reference for the interpre-

tation of antimicrobial susceptibility of fish bacterial pathogens (Alderman and Smith 2001). However, there is still a complete lack of data for acceptable MIC values of bacterial pathogens acting as control strains.

Table 7 contains the NCCLS standards for OT and SXT and Table 8 provides the number of resistant isolates observed in this work according to such standards.

Table 7. NCCLS MIC (serial dilution) interpretative standards (μg/ml) for OT and SXT.

| | Categories | | |
|---|---|---|---|
| Antibiotic | Susceptible | Intermediate | Resistant |
| OT | ≤ 4 | 8 | ≥ 16 |
| SXT | ≤ 2 | 4 | ≥ 16 |

Table 8. Number of OT and SXT aeromonads isolates categorised according to the MIC observed in this study

| | Categories | | |
|---|---|---|---|
| Antibiotic/Isolates origin | Susceptible | Intermediate | Resistant |
| OT – Singapore | 4 | 3 | 39 |
| OT – South America | 4 | 0 | 8 |
| SXT – Singapore | 8 | 13 | 25 |
| SXT – South America | 7 | 1 | 4 |

The majority of the isolates from Singapore (39) and South America (8) were above the resistant limit MIC value for oxytetracycline. This was expected as, during a previous study using the antibiotic disc diffusion test (del Rio-Rodriguez and Turnbull 2002), most of the isolates from both geographic sources were resistant to OT. With respect to SXT, 25 out of the 46 isolates from Singapore and 4 out of the 12 isolates from South America were resistant. However, most of the isolates from South America were susceptible to this drug and 13 and 8 isolates from Singapore fell into the category of intermediate and susceptible, respectively. Intermediate resistance is an important category in clinical practice, since this category "implies clinical applicability in body sites where the drug is physiologically concentrated, or when high doses of drug can be used" (NCCLS 1992). This suggests that SXT should still be a useful drug for the treatment or preventive treatment of imported ornamental tropical fish regardless of their origin.

Some studies have reported the maximum and minimum MIC values obtained for *Aeromonas* spp. known to be pathogenic to fish. Table 9 includes the guidelines for grading strains of *A. salmonicida* for sensitivity to OA, suggested by Tsoumas *et al*. (1989). Table 10 includes minimum and maximum MIC values reported for *A. salmonicida* and *A. hydrophila*. MIC results for the motile aeromonads have been included in tables 9 and 10 for comparison.

Table 9. Guidelines suggested by Tsoumas et al. (1989) for grading *A. salmonicida* sensitivity to OA and the frequencies of motile aeromonads from this study per category

| | OA Guidelines (μg/ml) | | |
|---|---|---|---|
| | Susceptible | Intermediate | Resistant |
| Origin | ≤0.062 | 0.125-0.5 | ≥1.0 |
| Singapore | 2 | 0 | 44 |
| South America | 6 | 4 | 2 |

Following Tsoumas *et al.* (1989), 44 isolates from Singapore and only 2 isolates form South America would be considered resistant. No isolate from Singapore would fall into the intermediate category and only 2 isolates would appear to be susceptible. Conversely, 4 and 6 isolates from South America would be categorised as intermediate and susceptible, respectively. The frequencies of the motile aeromonads in those categories reinforce the results observed during the antibiotic disc diffusion tests. Most motile aeromonads from Singapore appear to be resistant to OA while aeromonads from South America still appear to be susceptible.

Some minimum and maximum OA MIC values reported for *A. salmonicida* and *A. hydrophila* are displayed in Table 10. MIC values for *Aeromonas* spp. from Singapore spread over the range containing the lowest and the highest MIC test result. This highest MIC test result of the range is probably one of the highest OA MIC values ever recorded for a motile aeromonad isolated from ornamental fish imports. On the contrary, the minimum and maximum OA MICs displayed by the motile aeromonads from South America are similar to those reported by Martinsen and Horsberg (1994) and Guerin-Faublee *et al*. (1996) for *A. salmonicida*. However, the highest MIC value of the South American aeromonads recorded in this study is 20-fold higher than that report-

ed by Saitanu *et al*. (1994) for *A. hydrophila*. Nevertheless, only one strain of aeromonad from South America had this value (5 μg/ml). Most of the South American isolates' MICs were in the OA lower concentration range, therefore likely to be susceptible. Since OA is authorised for use in fish in the UK and since bacterial strains isolated from fish imported from South America into the UK have shown good sensitivity to this antibiotic, OA could provide a useful treatment for fish imported from South America, if required.

Table 10. Some OA and ENRO minimum and maximum MIC values (μg/ml) reported for *A. salmonicida* and *A. hydrophila* (in grey) in scientific literature and including data obtained in the present work

| Source | Range for OA | Range for ENRO |
|---|---|---|
| Inglis and Richards 1991 | 0.04 - 2.5[a]<br>0.04 - 10[b] | ≤0.005 - 0.3[a]<br>0.05 - 1.25[b] |
| Saitanu *et al*. 1994 | 0.025 - 0.4 | 0.002[c] |
| Martinsen *et al.* 1994 | 0.01 - 4.48* | 0.005 - 0.80 |
| Guerin *et al.* 1996/Bowser *et al* 1994 | 0.023 - 6 | 0.016 - 0.125 |
| *Aeromonas* spp. Singapore | ≤0.005 - ≥80 | ≤0.005 - 5 |
| *Aeromonas* spp. South America | 0.02 - 5 | ≤0.005 - 0.04 |

[a]7 isolates in first batch tested; [b]28 isolates in second batch tested
[a]7 isolates in first batch tested; [b]28 isolates in second batch tested
[c]only value reported
*Recorded at 15 °C

No guidelines for sensitivity to ENRO by *Aeromonas* spp. have been proposed. In this study, it was observed that at least one isolate from Singapore had a MIC higher than the values reported for *A. salmonicida* (Table 10). The MIC of the Singaporean isolate was 2-fold higher than the MIC reported for *A. salmonicida* by Martinsen and Horsberg (1994) and Inglis and Richards (1991) and 6-fold higher than the MIC reported by Bowser *et al.* (1994) for the same bacterium. It is also 12-fold higher than the value reported for *A. hydrophila* by Saitanu *et al*. (1994). In veterinary medicine, 8.0 μg/ml is one of the highest ENRO MICs reported for a Gram negative bacterium (*Pseudomonas aeruginosa* isolated from a dog, Green and Budsberg (1993)

cited in Hooper and Wolfson, 1993) and most Gram negative ENRO MICs are not higher than 1.0 to 2 μg/ml (Green and Budsberg (1993)). Consequently, the MIC value reported for this particular aeromonad isolate from Singapore could be considered unusually high among the aeromonads.

It is worth highlighting that in a previous paper (del Rio-Rodriguez and Turnbull, 2002), a small number of isolates were considered resistant to ENRO based on the disc diffusion test results. The MIC range observed for the Singaporean aeromonads was higher than that displayed by the South American aeromonads. There is some evidence that ENRO MIC ranges of different strains resistant to oxolinic acid are higher than the ENRO MIC ranges of isolates sensitive to OA (Martinsen *et al.*, 1992). This characteristic is indicated by a spread of MIC values over a range tested, and it has been suggested as an indication of possible cross-resistance (Inglis and Richards, 1991; Martinsen *et al*., 1992). Considering that 44 Singaporean aeromonad isolates showed resistance to OA, and were also spread over the ENRO MIC range tested for, it suggests that ENRO resistant aeromonads are emerging in the ornamental fish trade from Southeast Asian sources. This might be the consequence of the increased use of OA, although is not known if ENRO is currently used in Southeast Asia for fish therapy.

It is unfortunate that diseases of ornamental fish, especially bacterial diseases receive minor attention from fish scientists. In the last four years, little information has been produced on antimicrobial resistance patterns of bacterial isolates of imported tropical ornamentals (Kleingeld *et al.,* 2001). The monitoring of the development and evolution of antibiotic resistance of opportunist bacterial pathogens may help to realistically assess the risk associated with these imports to the trade itself. The threat posed by these bacteria to British native fauna or aquaculture animals is probably low, since ornamental tropical fish are normally kept in enclosed facilities. ENRO MIC values of Singaporean aeromonads matched moderate MIC values of pathogenic *A. salmonicida*. Due to the increasing importance of new quinolones in fish therapy, further work is needed in order to assess quinolone susceptibility of motile aeromonads accompanying ornamental tropical fish imports.

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PROCEEDINGS

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# Microscopic studies of the link between salmonid proliferative kidney disease (PKD) & bryozoans

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*Abstract*

*Proliferative kidney disease is a significant financial burden to freshwater salmonid culture in the UK. This paper describes studies of the development of the causative agent,* Tetracapsuloides bryosalmonae *and another related malacosporean parasite within their invertebrate freshwater bryozoan hosts and discusses possible control methods.*

Proliferative kidney disease (PKD) is an economically important parasitic condition which costs the UK trout industry approximately £2.5 million per annum (Feist 2004) and primarily affects first season freshwater salmonid fish in areas of Western Europe and North America (Clifton-Hadley and others 1984; Hedrick and others 1993). The parasite that causes PKD was originally known as PKX, denoting its uncertain taxonomic position in the phylum Myxozoa Grassé 1970 (Seagrave and others 1980). Subsequent studies discovered that freshwater bryozoans (Fig. 1) – known colloquially as "moss animals" - acted as additional hosts, and the parasite was eventually named *Tetracapsuloides bryosalmonae* and placed in the new myxozoan class Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000 (Anderson and others 1999; Canning and others 2002).

The disease is seasonal, typically occurring between May and September, with increasing water temperatures exacerbating the development of PKD in affected fish (Ferguson and Needham 1978; Foott and Hedrick 1987). Characteristic disease signs include renal swelling caused by a granulomatous hyperplasia that encompasses interstitial extrasporogonic *T. bryosalmonae* cells (Ferguson and Needham 1978). PKD leads to increased production costs with levels of morbidity of usually 100% and mortality ranging from below 20% in uncomplicated cases, up to 100% in fish suffering from secondary disease (Ferguson and Ball 1979; Clifton-Hadley and others 1986). Fish that have recovered from clinical disease exhibit apparent resistance to future *T. bryosalmonae* challenge (Ferguson and Ball 1979; Klontz and others 1986).

Various control methods have been developed for PKD, with varying levels of success. Husbandry measures - including lowering summer water temperature (using bore-hole water), delaying transfer of naïve stocks to endemic waters, eliminating secondary pathogens, reducing feeding rates and delaying grading - have been implemented as attempts to limit economic losses (Bucke and others 1981). Malachite green, the antibiotic fumagillin DCH and its synthetic analogue TNP-470 have been used therapeutically with some efficacy, but concerns over toxicity to fish, residue levels and environmental issues have prevented wide adoption of these treatments (Morris and others 2003). The perceived specific immunity that previously exposed fish demonstrate to *T. bryosalmonae* has led to interest in the possibility of developing a vaccine to combat the condition, although no such product is currently available.

During this study, laboratory culture techniques were developed to allow continuous maintenance of the bryozoan hosts of *T. bryosalmonae*. These filter-feeding colonial invertebrates are normally sessile, being adhered to submerged substrates such as wood and plastic. Bryozoans were collected from a loch, germinated from their overwintering stages (statoblasts) and maintained in aquaria. A feeding trial was undertaken, which gauged the nutritional value of more than 50 species of commercially cultured protozoa and algae for the bryozoans. These monocultures were added individually to Petri dishes containing the bryozoans, which were then observed under a dissecting microscope for their ability to ingest the experimental diet. Subsequent examination of released bryozoan faecal pellets assessed the level of digestion of those species consumed. It was found that while certain species were readily ingested, they were not digested with organisms surviving passage through the digestive tract, thus negating any nutritional benefit to the bryozoan. However, a selection of algae and protozoa were found to be equally readily ingested and digested by the bryozoans, presumably proving beneficial to their maintenance. No single species of algae or protozoa was identified which could maintain the bryozoan colonies alone, so mixtures of digestible cultures were used for the long-term maintenance of bryozoans.

Field trips were made to trout farms endemic for PKD in the south of England, and bryozoan specimens were collected from the inlet waters. Within several weeks of culture in the laboratory, the bryozoans revealed characteristic signs of myxozoan infection in several colonies. Two distinct parasitic infections were seen: *Buddenbrockia plumatellae* (Fig. 2) and *T. bryosalmonae* (Fig. 3), the former producing worm-like spore sacs while

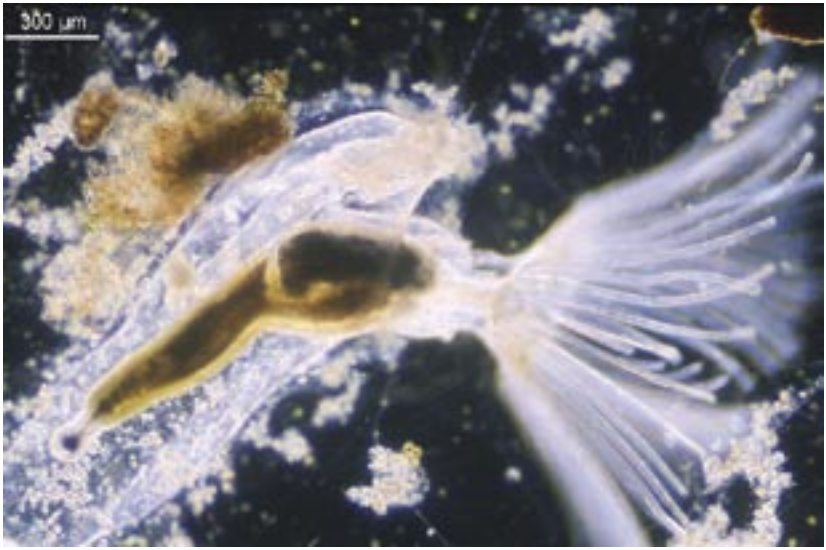


FIG 1: *Plumatella* sp. which has been fed on the algae *Haematococcus lacustris*.

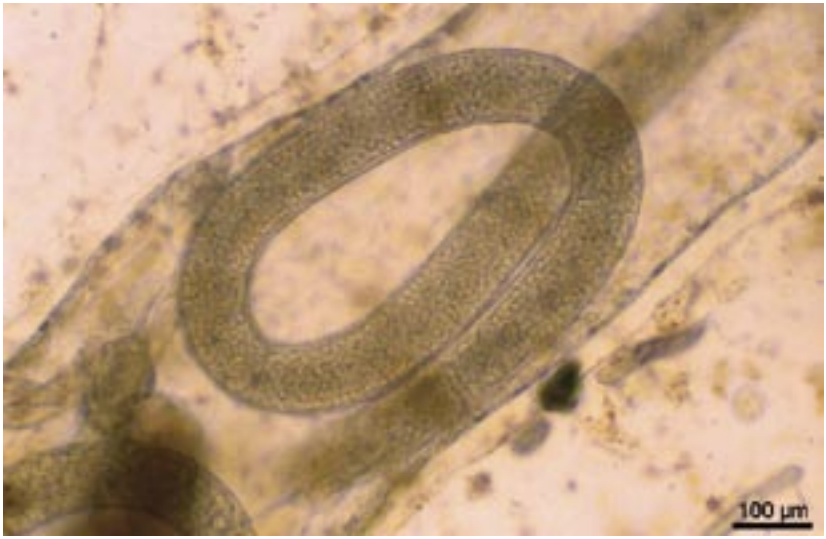


FIG 2: Coiled vermiform myxozoan parasite *B. plumatellae* within *P. repens*.

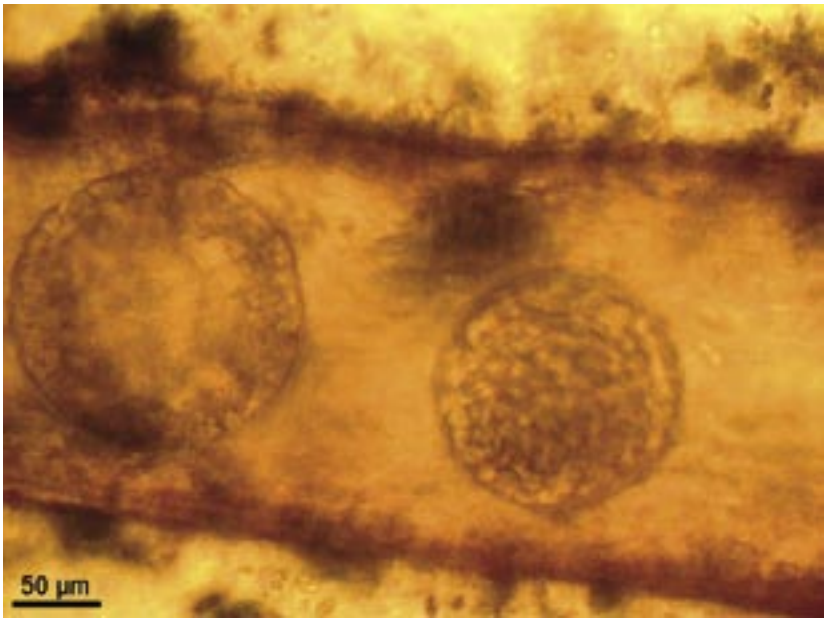


FIG 3: Spore sacs of *T. bryosalmonae* within *F. sultana*.

the latter developed spherical spore sacs. The ensuing development of these parasites was observed under an inverted microscope. Both still and video images were captured, demonstrating the movement of the parasitic developmental stages within the coelomic fluids of the bryozoan host.

With both myxozoan infections, the first observable sign of infection was the presence of numerous microscopic bodies swirling within the bryozoans. After one or two days, larger irregularly shaped bodies formed. In the case of *B. plumatellae* these adhered to the internal wall of the host, while with *T. bryosalmonae* they were free of attachment. Subsequently, the stages enlarged: *B. plumatellae* forming elongated oblong masses, while *T. bryosalmonae* infection led to formation of spherical bodies. Over the period of about a week, mature spore sacs were seen to develop. The vermiform spore sacs of *B. plumatellae* seen within the bryozoan *Plumatella repens* were up to 2 mm in length, apparently writhing independently of their host. Mature spore sacs of *T. bryosalmonae* within *Fredericella sultana* were of approximate diameter 100 – 200 µm and were seen to rotate and flow in the currents within the coelomic cavity of the bryozoan host. Upon maturation

of the sacs, they burst releasing numerous spores into the bryozoan, which were subsequently ejected into the surrounding water. The spores of both parasites were approximately 20 µm in diameter, each possessing the four characteristic polar capsules of malacosporeans.

To allow further examination, spores of *T. bryosalmonae* were dissected from infected colonies of *F. sultana*. The samples were then stained either with a fluorophore which specifically targeted certain polysaccharides or subsequently with dyes targeting lipid and nucleic acid cellular components. The spores were then examined under an inverted microscope attached to a Leica confocal laser scanning microscope and serial sections were captured and processed using a personal computer running Leica Confocal Software. The software allowed reconstruction of the images, leading to the production of three-dimensional representations of spore morphology (Fig. 4). It was found that the spores were regular in shape, having four capsular cells

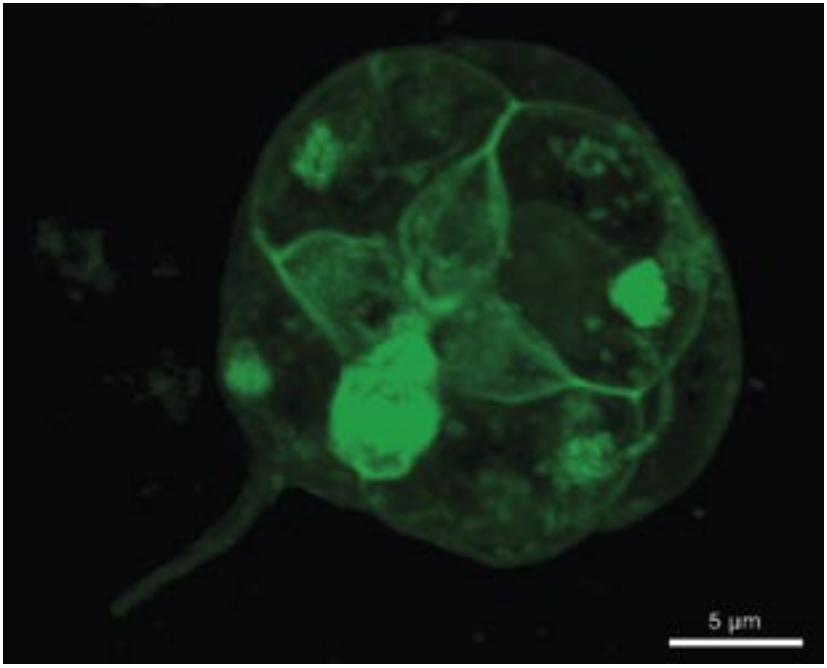


**FIG 4:** 3D reconstruction of a spore of *T. bryosalmonae* from confocal microscopy scans. Four central capsular cells (from one of which a polar filament has extruded) are encompassed by nucleated valve cells. (polysaccharide stain)

which included spherical polar capsules, surrounded by structural valve cells encompassing two central germinative sporoplasms. In the examination of samples following minimal processing, this work complemented previous ultrastructural studies of the morphology of *T. bryosalmonae* within bryozoan hosts (Canning and others 2000).

Infected bryozoan colonies were seen to thrive less well than unaffected regions. Waves of infection were seen within *B. plumatellae*-infected bryozoans, with a variety of developmental stages seen within individual colonies simultaneously. Fragmentation was a common feature in bryozoan colonies infected with malacosporeans, resulting in formation of numerous autonomous units. Although statoblast formation was noted in infected colonies, it was at a lower level than in comparable uninfected colonies. As statoblasts represent the only overwintering stage of most freshwater Bryozoa - except those of the genus *Fredericella* which can overwinter as colonies - this development may present a means of maintenance of parasites from one season to the next.

Rainbow trout (*Oncorhynchus mykiss*) were experimentally affected by PKD following exposure to material released from bryozoans infected with *T. bryosalmonae*, but similar exposure to *B. plumatellae* material did not result in noticeable disease. Further experimental trials have allowed quantification of the lowest dose of *T. bryosalmonae* spores that elicited PKD in rainbow trout, demonstrating the highly infective nature of the parasite. No evidence of horizontal transmission of myxozoan infection between bryozoan colonies was obtained – despite prolonged cohabitation trials and injection of material between colonies using micromanipulation techniques - supporting the theory that the parasites rely on other hosts (such as teleosts) to complete their life cycles.

The successful laboratory culture of infected bryozoan colonies seems crucial in furthering our understanding of PKD. This goal would increase the potential of discovering the missing links in the life cycles of these significant malacosporean parasites. The translucent nature of bryozoan colonies cultured in the laboratory system presented an exciting opportunity to observe myxozoan parasite development directly within a static host. Also, laboratory year-round maintenance of *T. bryosalmonae* could further allow controlled infection models to be developed without relying upon seasonally available bryozoan material. The observation that very high loads of *T. bryosalmonae* spores were released from bryozoans, in

combination with the experimental findings of the infectivity of the spores implied that very limited volumes of infected bryozoans positioned in the inlets to farms could potentially infect large numbers of farmed salmonids. This would mean that any attempt at bryozoan control as a husbandry measure to curb PKD would have to effectively eradicate all bryozoans in a waterway: practically very complex to achieve. Therefore, future control methods may rely on limiting stress, exposure to spores and the development of vaccination strategies.

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PROCEEDINGS

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# Analysis of a short tail phenotype in farmed Atlantic salmon (*Salmo salar*)

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*Abstract*

*The short tail phenotype represents one of the main causes for downgrading of farmed Atlantic salmon (*Salmo salar*) at slaughterhouses. Prevalence of short tail is variable and the aetiology is suspected to be multi-factorial. Risk factors have been identified but descriptions of the aetiology and the pathology of the condition are still rare. In the current study, a radiological and histological analysis of short tails has been performed, examining six normal and six downgraded individuals from a slaughterhouse in southern Norway. In the short tail phenotype, vertebral bodies were shifted and bent at the contact zone of adjacent vertebral bodies. Changes either affected the entire spine or were located at the medial caudal-spine. While the internal bone structure of the vertebrae was similar in deformed and non-deformed animals, a lack of intervertebral space apparently caused a shortening of the vertebral column and corresponded to an elevated condition factor in deformed individuals. Histological analysis revealed different degrees of proliferation of cartilaginous tissues, which replaced the intervertebral notochord tissue. The displacement of adjacent vertebral bodies and the development of cartilage in between vertebral bodies suggest mechanical forces as a possible cause for the observed deformations, since mechanically-induced overload and a subsequent direct contact of bones are factors that can stimulate heterotopic cartilage development and pseudoarthrosis.*

## Introduction and case history

Spinal deformity is one of the main causes for downgrading of farmed Atlantic salmon (*Salmo salar*) at slaughterhouses. One of the most frequently observed examples of spinal deformation is a condition referred to as "short tail". In short tail fish, the external shape of the fish is altered, the body length is decreased and the body height is increased. Accordingly, the condition factor is elevated and can reach values above 2 in severe cases. Pathological alterations can affect the entire spine or can be localised in the medial-caudal spine. The prevalence of short tail is variable and can range from 0 to 60–70% in different batches of fish. In this particular investigation, 13.3% of the fish were affected and consequently downgraded for this condition.

The aetiology of spinal deformity is suspected to be multi-factorial (Vågsholm and Djupvik 1998). Spinal deformity represents a group of pathologies in which genetic (Aulstad and Kittelsen 1971; Sadler *et al*. 2000; McKay and Gjerde 1986), congenital, toxicological (Bengtsson 1975; Couch *et al*. 1977; Wells and Cowan 1982), microbiological (Kent *et al*. 1989; Kvellestad *et al*. 2000) nutritional, husbandry (Halver *et al*. 1969; Roy *et al*. 2002; Kihara *et al*. 2002; Lewis *et al*. 2004) and environmental factors (Bæverfjord *et al*. 1996) might have a role in predisposing or determining the condition.

The documentation of this pathological condition from radiological and histological perspectives are rare (Kvellestad *et al*. 2000; Witten *et al*. 2005) and therefore additional descriptions and analysis of this condition are required in order to develop a greater understanding of the nature and origin of the problem.

## Objective

The objective of the study was to characterise alterations of the vertebral column in short tail farmed Atlantic salmon (*Salmo salar*) by radiological and histological examination.

## Materials and methods

Samples were obtained from a commercial slaughterhouse in southern Norway in November 2001. Six fish with a "normal" appearance (graded in superior category) and 6 downgraded fish with an obvious "short tail" phenotype were selected. Body weight and length was measured in all indi-

viduals. Condition factor (CF) was calculated as:

$$CF = [Weight\ (g)/\ fork\ length\ (cm)^3] . 100$$

X-rays of the spine and bone samples for histological examination were taken.

## Radiology

X-rays from all fish were taken using a portable Mini X-ray HF80+ machine (Mini X-ray inc., Northbrock, USA) and Kodak Industrex M Film Ready Pack II (Kodak Industry, France). No screens for increasing the strength of the X-ray beam were used. The setting of the X-ray unit was 70 kV, 15 mA, 2 sec exposure time, with a distance of 40 cm between the beam source and the X-ray film. Radiographs were developed with Kodak chemicals according to the protocol of the manufacturer (Witten and Hall 2002).

## Histology

Vertebrae were fixed in 10% neutral buffered formaldehyde for 24 hrs, rinsed in tap water for 24 hrs, and decalcified for 72 hours in a 10% EDTA solution buffered with 0.1 M TRIS base at pH 7.0. After decalcification, samples were stepwise dehydrated and embedded in Paraplast.

Ten µm serial sections were prepared in the sagittal plane of the vertebral columns, starting at the periphery and ending in the middle plane of the vertebrae. Sections were stained with Masson's trichrome as the basic analytical procedure according to the protocol used by Witten and Hall (2003) for salmon bone.

## Results and discussion

Compared to normal fish, deformed animals displayed a pronounced short tail phenotype (Fig 1), with a reduced fork length, an increased weight, and an elevated condition factor (Table 1). X-rays revealed the type of vertebral deformation in comparison to non-deformed animals (Fig 2). A lack of inter-vertebral space was related to the shortening of the vertebral column (Fig 3). In addition, deformed animals showed inward bending of the vertebral edges in the contact (and growth) zone of adjacent vertebrae and the displacement of vertebral bodies (Fig 4). In normal shaped animals single vertebrae can be affected, but in animals with pronounced short tail phenotype, almost the entire vertebral col-

FIGS 1–10 (opposite)

FIG 1. Full-grown non-deformed (N5) and deformed (D6) Atlantic salmon. Deformed animals displayed a pronounced short tail phenotype with a reduced fork length and an elevated condition factor.

FIG 2. Radiograph of regularly shaped and spaced vertebral bodies of a non-deformed animal. Bar = 1.5 mm.

FIG 3. Radiograph of a partly deformed vertebral column showing that deformation can affect one side of a vertebral body (black asterisk) without affecting the other side (white asterisk) or neighbouring vertebral bodies. A lack of inter-vertebral space (black asterisk) is related to the shortening of the vertebral column (red arrows). The white asterisk indicates a normal inter-vertebral space. Bar = 1.5 mm.

FIG 4. Radiograph of homogeneously deformed vertebral bodies. Deformed vertebrae show inward bending of the vertebral edges in the contact (and growth) zone of adjacent vertebrae and the displacement of vertebral bodies (white arrowheads). Bar = 1.5 mm.

FIG 5. Non-deformed animals display a regular vertebral growth/contact zone with bone forming cells (black arrowhead), newly formed bone (white arrowhead), a notochord sheath (white asterisk) and a notochord (black asterisk). Masson's trichrome staining. Bar = 250 $\mu$m.

FIG 6. Intervertebral space of two deformed vertebral bodies (see Fig. 4, white arrowheads). The notochord sheath has disappeared and is now replaced by cartilage (black asterisk). Masson's trichrome staining. Bar = 250 $\mu$m.

FIG 7. A higher magnification of the intervertebral space of two deformed vertebral bodies displays osteoblasts (black arrowhead), bone (white asterisk), hyaline cartilage (black asterisk), and transitional stages between bone and cartilage. Masson's trichrome staining. Bar = 150 $\mu$m.

FIGS 8 and 9. Compared to notochord tissue in the intervertebral space of a non-deformed animal (Fig 8), remaining notochord tissue between deformed vertebral bodies displays an altered structure (Fig 9). Masson's trichrome staining. Bar = 250 $\mu$m.

FIG 10. The internal vertebral bone structure was found normal and solid in both groups of animals (deformed and non-deformed) with no signs of under-mineralization, impaired bone growth, micro-fractures or enhanced resorption. Masson's trichrome staining. Bar = 250 $\mu$m.

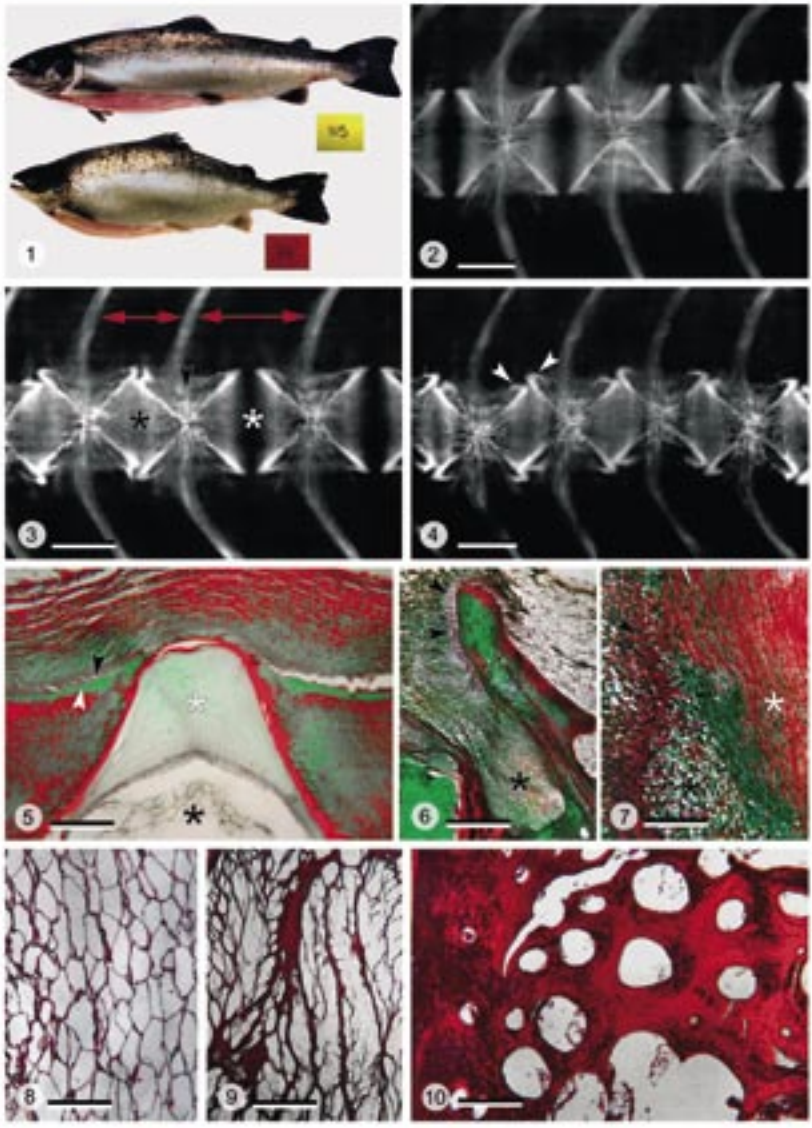
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TABLE 1: Comparative mean size and condition of deformed and normal fish

| | Normal (superior category) n=6 | Short Tail (downgraded) n=6 |
|---|---|---|
| Fork length (cm) | 61.2 ± 1.9 | 55.3 ± 2.8 |
| Weight (kg) | 2.4 ± 0.2 | 2.8 ± 0.3 |
| Condition factor | 1.1 ± 0.1 | 1.6 ± 0.1 |

umn was deformed (Figs 3, 4). At the histological level, non-deformed animals displayed a regular vertebral growth/contact zone with bone forming cells, newly formed bone, a notochord sheath, and a notochord (Fig 5). In deformed vertebral bodies, the notochord sheath had disappeared and was replaced by cartilage (Fig 6). A higher magnification displays osteoblasts, bone and hyaline cartilage. Also, transitional stages between bone and cartilage were observed (Fig 7). Remaining notochord tissue in the intervertebral space of deformed animals also displayed alterations (Figs 8, 9). The internal vertebral bone structure was found normal and solid in both groups with no signs of under-mineralization, impaired bone growth, micro-fractures or enhanced resorption (Fig 10). These findings were supported by the results of a bone mineral analysis, not shown in this paper, that yielded similar results in both groups.

The short tail phenotype has been linked to shortening of the vertebral bodies themselves, as well as to the fracture and fusion of vertebral bodies (Vågsholm and Djupvik 1998; Kvellestad *et al*. 2000; Gavaia *et al*. 2002). In the present study, the primary reason for the shortening of the vertebral column appeared to be related to alterations of the tissue of the inter-vertebral space and of the vertebral growth zone. The regularly shaped bone of the vertebral body spongiosa, however, suggests that the animals did not suffer from a lack of minerals or a disorder of mineral metabolism. Similar results were obtained by Witten *et al*. (2005), examining a short tail phenotype with vertebra bodies that were homogeneously deformed but, unlike this case, not displaced.

The displacement of adjacent vertebral bodies and the development of cartilage between vertebral bodies raises the possibility that mechanical forces could have caused the observed alterations. Mechanically-induced overload and a subsequent direct contact of bones are factors that can stimulate heterotopic cartilage development and pseudoarthrosis (Shih *et al*. 2001). In salmon, mechanical forces and the expression of local growth factors are suggested to be responsible for switching from bone formation to cartilage formation (Witten and Hall 2003) in the jaws of juvenile and adult wild

Atlantic salmon (Gillis *et al*. 2004; Witten and Hall 2002; Witten *et al*. 2004). The shifting of the vertebral bodies and the heterotopic development of cartilage are clear indications of the disorder described herein. Developing this line of thought, the short tail phenotype could primarily relate to alterations of the intervertebral tissue and bone alteration may be only a secondary phenomenon. Consequently, focusing on alterations of the intervertebral tissue could enable the early detection of this type vertebral malformation.

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# Management of *Saprolegnia* on the farm – a veterinarian's view

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*Abstract*

*Saprolegnia infections are common in freshwater salmonid culture and successful management depends upon an understanding of the pathogen, host responses, risk factors and methods of control. Therapeutants have a part to play but close attention must be paid to those aspects of husbandry which increase the risk of infection and clinical disease.*

## Introduction

Fungal diseases of fish have been a problem for as long as fish have been cultured and are especially important to fish farmers. In freshwater salmonid production, the ability to control fungus, (particularly *Saprolegnia*), is an essential part of good husbandry and welfare, and it is worth reviewing where we are today.

## What is *Saprolegnia*?

The most important pathogenic aquatic fungi are members of the Oomycetes (water moulds) and these, particularly the *Saprolegniaceae*, are by far the most significant pathogens of eggs and fish. The Oomycotea belong to the same group as the algae but lack the photosynthetic pigment chlorophyll and so exist as saprophytes or parasites. In fact the word 'fungus' is probably incorrectly applied to members of the *Saprolegnia* family which are better described as pseudo-fungi. This difference is not just academic since potential therapeutics targeted at true fungi may be ineffective when applied to the treatment of *Saprolegnia*.

The identification of fungus commonly found on salmonid eggs and fish is not an easy matter and there is little agreement on how the pathogen should be named. However for all practical purposes it is common now to refer to

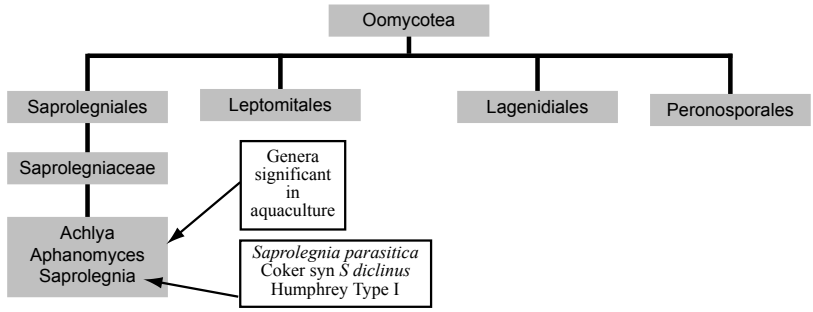



FIG 1: Taxonomy of *Saprolegnia*

the *Saprolegnia diclina-Saprolegnia parasitica* complex, but this article will refer simply to *Saprolegnia*. Other species of *Saprolegnia* and related fungi such as *Achlya* may also colonise eggs and fish reflecting the composition of the local flora.

*Saprolegnia* is a filamentous organism found in freshwater and moist habitats. It is nourished by feeding on dead organic material, but some strains are opportunist and can parasitise living animals including salmonid eggs

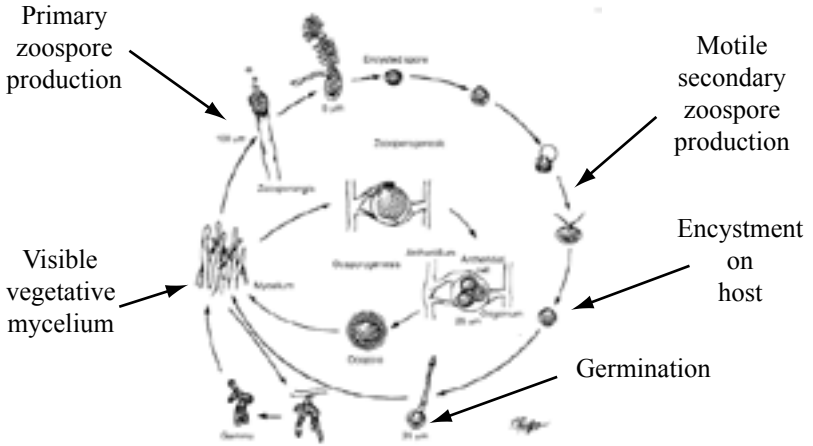



FIG 2: Diagrammatic representation of life cycle of *Saprolegnia*

and fish. The result of this is immediately recognisable to any farmer as the appearance of white cotton-wool-like tufts which if uncontrolled can smother and kill large numbers of eggs and fish.

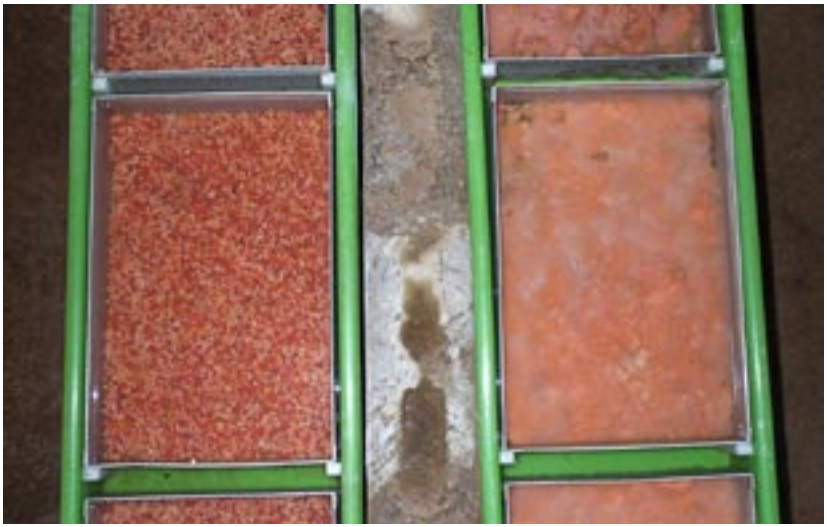
The asexual propagation stage of *Saprolegnia* is the zoospore, a free-swimming motile stage, which is produced from the familiar filamentous hyphae, into surrounding water. Zoospores encyst in a protective envelope and can survive for long periods in the environment. Secondary zoospores can remain active for extended periods and act as the main dispersion stage for the pathogen. There is evidence that they are attracted to suitable hosts by chemotaxis and pathogenic isolates germinate rapidly in the presence of a host. The challenge is present at most times of the year and therefore the need for control is ever-present.

## How does Saprolegnia affect eggs and fish?

All life stages of salmonids (and many non-salmonids) in freshwater are susceptible to *Saprolegnia*. The pathogen has a global distribution and can even occur in marine species. In incubators, dead eggs form a very suitable organic substrate for the settlement of fungal spores and subsequent hyphal growth. Viable eggs seem able to resist colonization up to a point but can be overwhelmed if the challenge persists uncontrolled. In extreme cases entire batches of eggs can be lost to *Saprolegnia* (Figure 3)

In low intensity systems dead eggs are easily identified and can be manually removed (picked) as they appear, to reduce the risk to living eggs. However this is not practical in most commercial systems, and in these situations it is necessary to regularly flush an effective (pseudo) fungicide through the incubator. The frequency of treatment depends on local conditions and challenge but may have to be a daily routine from fertilisation until hatching. After the eyed stage it is of course possible to handle eggs to remove dead eggs, thus reducing risk.

Fish in unprotected waters are likewise subject to varying degrees of challenge but the risk factors associated with clinical disease are rather more complex and depend not only on natural factors, season, temperature, environmental conditions, but also on stressors created by the farming process itself. *Saprolegnia* is a versatile pathogen and can affect a number of organs according to host species e.g. the nervous system (farmed pike), the cornea (Indian glass barb).



Trough 1 Trough 2

FIG 3: Trough 2 showing severe loss of eggs due to *Saprolegnia*.

In the earliest stages of infection, colonization is often centred on the fins rather than the body and this may reflect the prevalence of fin erosion in crowded conditions. Infection of salmonid fry may not be overt and easily identified as a cause of loss. In these small fish the fungus is located initially on the gill filaments, intestine or yolk sac. A hyphal mass may develop in the pharynx preventing feeding and leading rapidly to starvation. Heavy infection of the gills may result in respiratory failure.

FIG 4: Adult salmon showing large areas of body affected by *Saprolegnia*.

On infected fish, *Saprolegnia* takes the form of profusely branched hyphae which grow to form the familiar cottonwool-like tufts or mycelia on the skin. Early lesions are grey-white in appearance and are often circular or crescent-shaped. Colonies extend outward, often at alarming speed, and coalesce with adjacent colonies. Large areas of the skin can be affected, with serious consequences for the individual (Figure 4).

The chance of recovery from *Saprolegnia* is directly related to the area of skin affected so prompt detection and effective treatment is vital.

Beneath the fungal mat, there is necrosis of the skin and haemorrhage, which, if left untreated, will result in death. The fish is affected in much the same way as a burn patient i.e. osmo-regulation fails. In catfish and Atlantic menhaden infection can cause severe dermal ulceration. `Winter kill` is a major cause of loss in channel catfish farming.

Given the continuous exposure to challenge and the potential virulence of *Saprolegnia*, how can fish survive at all in the freshwater phase?

Fish possess an immune system similar in many ways to the mammalian one and which operates at several levels. This means that a pathogen, be it fungal, bacterial or viral, has to overcome those defences to cause clinical disease, and healthy uncompromised animals can resist challenge. What are the defence mechanisms by which fish protect themselves and how are these mechanisms breached? *More importantly what can be done to mitigate the risk in commercial conditions?*

Since fungal spores colonize the skin primarily, this is the first line of defence. The integument of fish forms a physical barrier to colonization by *Saprolegnia*. The epithelial layer and the mucus it produces form an effective defence in the healthy fish and the skin is thought to produce anti-fungal chemicals which contribute to successful defence under normal circumstances. However it is primarily as a physical barrier that the skin resists colonization. A major reason that fungal lesions fail to develop on healthy fish is that germinating zoospores are shed naturally with continual loss of mucus from the skin surface. *Any damage to the skin facilitates infection and is clearly a predisposing factor to disease.*

In addition there are non-specific and specific internal defence mechanisms. *Factors which undermine the immune response increase vulnerability to disease.* One such factor is the presence of another disease that can increase vulnerability.

Temperature also plays a part in determining clinical outcome. Most metabolic processes are slower at low temperature and this includes wound healing, so fish subject to damage are more easily colonized. *Saprolegnia* seems capable of growth across the range of temperature in Scotland and some strains grow better as temperature increases. Thus the risk exists at all times of the year.

In aquaculture, there are a number of circumstances under which susceptibility to clinical disease is markedly increased. These may arise as a result of the physiological status of the fish, e.g. smoltification or because of external man-made factors, e.g. vaccination.

- Sexual maturation is associated with a marked increase in susceptibility particularly in male fish. This is not just associated with seasonal factors but with the hormonal changes occurring at this time.

- During smoltification a number of hormonal changes prepare the fish for existence in seawater and fully smolted fish held back in freshwater are particularly at risk.

- Any handling procedure may be accompanied by skin damage and increased susceptibility

- Vaccination presents particular risks and the whole process should be reviewed regularly to ensure best practice

- Adverse environmental conditions predispose fish to a wide variety of diseases including *Saprolegnia*. These may include social, physical or chemical factors, some resulting from sub-standard husbandry.

- Other concurrent disease

There is a factor common to these circumstances and that is the production by the fish of the hormone cortisol, the so-called `stress hormone`. Cortisol plays an important and positive physiological role during periods of stress but when the event is prolonged leading to chronically elevated cortisol, a number of undesirable consequences arise. Principal amongst these are partial suppression of defensive immune responses and inhibition of wound healing. These are the very processes upon which fish depend to resist colo-

nization by *Saprolegnia* and *efforts must be directed towards reducing the magnitude and duration of the stress response.*

## **Managing** *Saprolegnia*

In order to effectively manage *Saprolegnia*, it is essential to identify risk and to take every measure possible to reduce it.

So what can be done? It is easy to talk about `reducing stress` and applying `high standards of husbandry` but what does that mean in practice?

Firstly and most importantly don't underestimate the danger of this very familiar pathogen which can rapidly overwhelm a population of eggs and fish. *Saprolegnia* management should be a primary consideration in husbandry rather than an afterthought to be dealt with when clinical disease appears. Put in place a treatment plan in advance of spawning since established infections are almost impossible to control.

For egg incubation facilities, risk reduction is a function of design i.e. such factors as:

- Exclusion/avoidance of the pathogen by water treatment
- Maintenance of adequate water flow to avoid accumulation of organic detritus
- The ease with which preventive treatment can be applied e.g. all incubation units fully and equally perfused with water and therefore any therapeutant used. Troughs are easier to manage than cylinders.

However the biggest single risk factor for saprolegniasis of eggs is poor egg quality. The following may compromise quality of salmon eggs:

- Over ripe broodstock
- Poor broodstock condition
- Prolonged transport of eggs with inadequate temperature control
- Spawning in seawater
- Inadequate hygiene at spawning

During the course of incubation, where *Saprolegnia* is present, risk increases with time. It is customary to delay sorting until the `eyed` stage, i.e. at 250-degree days, on the grounds that eggs are too fragile to subject to the trauma of mechanical handling. However experience from the field suggests that it

may in fact be possible to bring forward sorting by up to 30 degree-days with the benefits that will bring in reducing challenge.

For fish it is important to bear in mind the cornerstone of *Saprolegnia* control i.e. to identify and manage those situations which *compromise the fish's own ability to resist disease.*

It is not difficult to draw up a list e.g:

- Reduce handling to a minimum and where it is unavoidable make every effort to prevent skin damage. It is worth considering wearing latex surgical gloves when handling fish, since the human hand is like coarse grain sandpaper to a fish
- Keep tanks clean and avoid overfeeding since waste feed is an ideal substrate for *Saprolegnia*
- Manage stocking density and feeding to minimise competition, stress and cannibalism
- Change processes which incur an unmanageable risk
- Don't hold back smolts in freshwater
- Monitor/control water quality in high density systems
- Identify and treat concurrent disease

It may be worth screening the incoming water supply for spore numbers on a regular basis to detect how ambient environmental factors affect the challenge. This information may be of value in planning a management strategy. A method can be found in *Fungi and Fish Diseases*, Chapter 6 (see recommended reading)

## Vaccination

The process of vaccination by injection deserves special attention since it is one of the most important husbandry factors pre-disposing to disease. It is an event traumatic to the fish and compromises all of the animal's protective defences making it very vulnerable to clinical *Saprolegnia*. This is illustrated in Figure 5.

Day 0 is the day of injection. Shortly after this point the defences of the fish fall `below the line` and the animal enters a period of vulnerability during which it is very susceptible to colonization by S*aprolegnia* spores. An overt indication of this is loss/suppression of appetite.

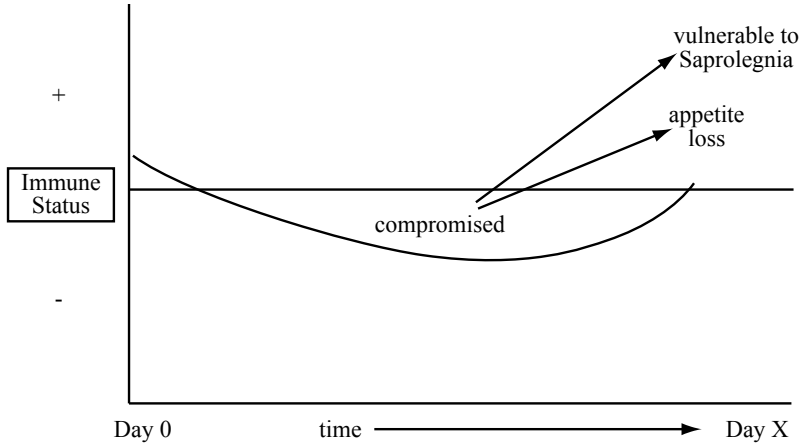



FIG 5: Graph showing period of vulnerability following vaccination.

The factors that determine the duration of this period of vulnerability are not certain but may be related to:

- The vaccine formulation
- Ambient temperature
- State of smoltification e.g. in light manipulated smolts
- Skill of vaccinators and suitability of equipment
- Degree of trauma during vaccination
- Level of challenge which varies from place to place and seasonally
- Intercurrent disease

Experience suggests that the period may last for up to 200-degree days.

Regular removal of dead fish after injection significantly reduces challenge by removing a source of zoospores and especially if this can be done with the minimum of trauma e.g. by using airlifts instead of `rolling` nets to recover dead fish. The vaccination table should be kept wet at all times to reduce the risk of trauma to the skin. The whole process should be critically evaluated to identify risk factors.

Vaccination plans should include a *Saprolegnia* risk assessment which takes into account all of the above with a view to reducing to a minimum the risk of disease. As with eggs it is essential to be prepared before the period of risk and also to make sure that other clinical conditions which may be present are identified and dealt with.

## Conservation hatcheries

There are many organisations engaged in salmonid conservation in the UK. Wild broodstock may be taken from rivers each year for stripping and reconditioned annually for up to five years. These kelts are of high individual value.

Progeny derived from wild caught broodstock are used in re-stocking schemes to enhance the fishery.

Egg incubation in conservation hatcheries is usually low density allowing regular removal of dead eggs thus preventing saprolegniasis. However broodstock are more problematic and require particular care.

Capture methods (electro-fishing, trapping, netting) can be particularly traumatic for fish which may already be damaged during migration. These fish are vulnerable to fungal infection not only because their natural defences are compromised, but also because their physiological condition predisposes them to colonization. A number of practical measures can help to reduce risk:

- Avoid holding fish for longer than is necessary. Try and capture fish only when they are close to spawning.
- Ensure that capture equipment is as atraumatic as possible i.e. less likely to damage the skin of the fish
- Select healthy individuals
- Since male fish are particularly vulnerable, take as few as is practical
- Keep males and females separate
- Consider taking and preserving milt at capture to avoid holding males at all
- Wear smooth gloves at all times when handling fish
- Treat wounds and secondary bacterial infections. Consider routine injection of a suitable long acting antibiotic but be aware of the legal requirement regarding medicines residues.

A thorough risk assessment should be carried out and practices modified to mitigate risk at all points in the process.

## Treatment of *Saprolegnia*

The use of therapeutants is just one piece of the jigsaw and should not be regarded as a prop to support husbandry of a less than perfect standard.

Treatment plans should be carefully devised in light of risk assessment and applied appropriately.

The only product authorised in the UK for the treatment and prevention of *Saprolegnia* in salmonids is Pyceze, a topical formulation of bronopol. Pyceze is a Prescription Only Medicine (POM) available only from veterinary surgeons.

Pyceze is indicated for the treatment and prevention of *Saprolegnia* in farmed fertilized salmonid eggs and in Atlantic salmon.

It is clear from initial clinical experiences that treatment of fish requires particular care and attention to detail. Pyceze is not persistent so it is only active against *Saprolegnia* for the period of exposure. This means that fish have to be protected (by treatment) for as long as they are compromised and vulnerable.

It is essential to formulate a treatment plan by site, based on assessment of the risk, and to adhere to the plan but be prepared to amend it based on clinical circumstances. Farmers and their prescribing veterinarian are best placed to carry out this exercise using knowledge of local conditions and up to date information from medicines suppliers.

## Recommended Reading

*Fungi and Fish Diseases* by L.G Willoughby. Pisces Press – Stirling (1994) ISBN 0 9521198 11

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# Expert Evidence and the Role of the Expert Witness – A practical Guide

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*Abstract*

*From a legal standpoint, veterinary surgeons play a unique and pivotal role in the care and welfare of animals. For this reason, at some point in a veterinary surgeon's career it is likely that he or she will be called upon to act as an expert witness and appear in court. Without some preparation and training, this can be a daunting prospect. This paper describes the special role of the expert witness, how to prepare and give evidence and also briefly describes the trial process.*

## Introduction

How would you feel if someone were paid to undermine your credibility, your professionalism, your expertise, your ability to analyse data, your ability to recall, your qualifications, and your experience? Welcome to the courtroom!

At some point in their career, a veterinary surgeon is quite likely to be asked to act as an expert witness and may have to give expert evidence in a court room. To help prepare a veterinarian for this role, this paper will deal principally with the following matters.

- What is expert evidence?
- The role of the expert witness.
- The trial process
- Giving evidence
- How to prepare.

## Expert evidence

Expert knowledge was of importance to the courts as far back as the fifteenth century and even earlier. This evidence was usually given in the form of a special jury empanelled to assist the court with specialist knowledge. From the fifteenth century until the seventeenth century there was increas-

ing acceptance of the practice of calling skilled experts to provide specialist knowledge. In a case in 1554, *Buckley* v *Rice,* Thomas, the judge stated:

> "If matters arise in our law which concern other sciences or faculties, we commonly apply for the aid of that science or faculty which it concerns, which is an honourable and commendable thing in our law."

Expert evidence is admissible if it is both relevant and probative.

Expert evidence will be admitted into court when the court has before it matters at issue, which require expertise for their observation, analysis or description.

## The Expert Witness

Expert witnesses are a special category of witness. The general rule is that a witness can only testify about something which he/she has seen, heard or felt and that mere opinions are irrelevant. The expert witness is an exception to this basic rule of evidence as the expert witness uniquely can give an opinion which is admitted as evidence in court.

## What is an Expert?

An expert is one who is expert or who has gained skill from experience.

It is for the court to decide whether the witness is so qualified as to be considered an expert. The court has a broad discretion to decide whether or not a person is capable of giving expert evidence. The two most important qualities are possession of knowledge and an ability to use that knowledge by virtue of training and experience.

Someone who is called as a witness of fact may also be tendered as an expert. For example a police officer with 15 years experience in traffic branch who had passed an examination in accident investigation and who had attended over 400 accident scenes was allowed to give evidence in court as an expert.

## Duties of an Expert Witness

The expert should be aware of the duties which he owes to the court. These were set out in the judgment of Cresswell J in *Compania Naviera SA* v. *Prudential Assurance Company Limited (the Ikarian Reefer) [1993] 2 Lloyds Reports.*

1. Expert evidence presented to the court should be, and should be seen to be, the independent product of the expert uninfluenced as to form or content by the exigencies of litigation.
2. An expert should provide independent assistance to the court by way of objective unbiased opinion in relation to matters within his expertise.
3. An expert witness should state the facts or assumptions upon which his opinion is based. He should not omit to consider material facts, which could detract from his concluded opinion.
4. An expert witness should make it clear when a particular question falls outside his expertise.
5. If an expert's opinion is not properly researched because he considers that insufficient data is available, then this must be stated with an indication that the opinion is no more than a provisional one.
6. If an expert cannot assert that the report contains the truth, the whole truth and nothing but the truth without some qualification, that qualification should be stated in the report.
7. If, after exchange of reports, an expert witness changes his view on a material matter having read the other side's expert's report, or for any other reason, such change of view should be communicated (through legal representatives) to the other side without delay and (where appropriate) to the court.
8. Where expert evidence refers to photographs, plans, calculations, analyses, measurements, survey reports or other similar documents, they must be provided to the opposite party at the same time as the exchange of reports.

## The Trial Process

For convenience, in relation to the trial process, law can be divided into two systems; adversarial and inquisitorial. The European Court of Justice (ECJ) is an example of an inquisitorial system where the emphasis is on the gathering of documentary evidence and the oral hearing is simply to tidy up detail.

In the adversarial system, characteristic of common law jurisdictions, the emphasis is very much on the respective strengths of each side, based on the oral arguments presented. This means that there are at least two versions of the same factual situation and both sides will present evidence to argue that their version is the right one.

The whole process can be compared to telling a story. Each side has their version. Lawyers structure cases in order to persuade. One of the most important aspects of the adversarial process is the use of witnesses to help present the story. Lawyers can also use documentary evidence and physical evidence, e.g. a weapon used in an alleged attack.

The judge is the decision-maker. As an expert witness you should bear this in mind at all times during the trial. So it is important to ensure that the judge receives your attention, courtesy and respect throughout the trial.

In Northern Ireland and England, there is a court list setting out the order in which cases are expected to be heard in both the County Court and High Court. There is a 'call over' at the beginning of the court day, when the list will be read out. At this stage if any cases are settled that fact will be announced. Counsel will announce their appearance at the beginning of the case.

Counsel for the plaintiff will open a civil case. Counsel will give a brief explanation of the background of the case, giving a brief chronological history of the fundamental events from which the case arises. The opening should highlight the issues in the case. Counsel will also hand into court any agreed documentation such as reports or photographs. A report is agreed when both sides indicate that they do not intend to challenge the contents of the report and are satisfied that it becomes evidence in the case.

Once a report has been agreed, the maker of the report is no longer required to give oral evidence to the court.

## Giving evidence

All evidence in court is sworn and you will be required to take an oath to tell the truth. If you object to swearing on the Bible, you should inform your counsel in advance and he/she will advise the clerk who will have the alternative affirmation ready. When taking the oath, you will stand and place a hand on the Bible. Simply repeat what the clerk says to you. Do not fidget. Use the oath to settle yourself; it will be the first time that you will hear your voice in the courtroom. Do not mumble; establish from the outset that you are in control.

The taking of the oath is a serious matter and there should be no noise in the court when an oath is being taken. Once you are sworn you will be asked to state your full name for the court.

In Northern Ireland and the Republic of Ireland, you will then be seated and you will give your evidence while seated (unlike England).

## Some useful legal terminology

The judge is referred to as:
'Your Worship' in the Magistrates Court
'Your Honour' in the County Court
'Your Lordship' in the High Court.
Barristers are known as 'Counsel for the Plaintiff' and 'Counsel for the Defendant'.

## Giving expert evidence in court

1. Be ready to state your professional qualifications at the beginning of your testimony. Have these ready; this will establish you as an expert witness and prevent any adverse comment from the opposing counsel who will want to challenge your opinion.
2. Direct all your answers to the judge. He is the decision-maker and you are there to assist him. The judge is taking a note of the evidence and you should watch the judge's pen to ensure that you are not speaking too fast. This will make the judge well disposed to you.
3. Keep a good professional composure.
4. Speak in a clear voice and at a steady pace. Watch the judge's pen - adopt a pace which allows him to write down what you are saying.
5. Do not feel pressurised. Never hesitate to ask for a question to be repeated.
6. Be professional; do not argue with counsel no matter how tempting.
7. Do not engage in conversation with counsel. Always refer your answers to the judge.
8. Never lose your temper.
9. Be patient. Counsel may ask you the same question in different ways to try to break you down. Be aware of the bigger picture and answer professionally. If there is to be any criticism of the method of questioning, that must be done by your client's representative or the judge.
10. Be HONEST. If you do not know, say so. Do not stray outside your area of expertise. Your credibility is at stake. Never give an answer simply because you think that is the answer counsel is looking for; always give a truthful answer.
11. Refresh your memory before you go to court by reading your report and your notes.

## Stages of Giving Evidence in court

**Examination-in-chief**
The interrogation of a person on oath is known as examination. In court the evidence of a witness is generally obtained by oral examination, called the examination-in-chief. The examination-in-chief is when your client's representative takes you through your evidence. The examination-in-chief should paint a detailed picture of the evidence of the case to the judge.

The purpose is to give all the relevant facts to the judge. It will also deal with issues which may arise in cross-examination and which are possibly the weaker parts of the case.

In the examination-in-chief it is important that you tell the judge all the relevant information. Scientific or complex matters should be explained accordingly. In the examination-in-chief, you will be asked about your report and may be asked to expand on it and explain it.

You should have an opportunity to meet with your client's representative before the trial to establish the important points of the case. Your client's representative when conducting the examination-in-chief cannot ask leading questions. These are questions which suggest the answer or assume certain matters, which are in dispute. Leading questions can be asked about introductory matters and matters which are not in dispute.

**Refreshing your memory**
It is permissible to re-read your report before you go to court. Once you give evidence, you may be referred to your report, which may be admitted to the court. You can also use notes to refresh your memory if the judge is satisfied that these were made contemporaneously.

**Cross-examination**
Cross-examination is when the opposing barrister asks you questions about your evidence for two main purposes:

1. He/she will want to undermine or qualify your evidence or diminish the effect of your evidence or reconcile your evidence with the evidence in their client's case.
2. He/she will want to put the defendant's case to you so that you can directly comment on it, e.g. "my client would say that you did not visit his premises on 4th October to inspect the conditions of the fish farm…"

In cross-examination, counsel can use leading questions.

Common areas of attack in cross-examination are

1. Your ability to accurately recall events (notes made at the time are helpful.)
2. Your analysis or opinion arising out of the facts. You are an expert witness and you rely on your experience and the relevant authorities on the subject and all of these are admissible to the court.
3. Your assessment
4. Your professional standing and credibility. Stay within your area of expertise. Do not bluff. Do not be afraid to state that you cannot comment on a particular area.
5. Reliability of observation.

**Useful Tips**

1. Be prepared. Know your case thoroughly and be familiar with the detail. Know your file and be able to refer to documents if you are asked to do so. Have original documentation if you have it on file. Know all the strengths of your case but also know the weaknesses and be ready to answer questions on grey areas in cross-examination. If you rely on photographs, make sure these can be proved in court, i.e. did you take them? If not can the photographer come to court to prove them? If anyone else has contributed to the report, check with counsel, as they may also be required to attend court.
2. Body language. Wear suitable clothing for court. Retain your composure and do not slump into the seat at an awkward question.
3. Listen carefully to what you are being asked. Seek clarification if you are not sure about what you are being asked. The judge will be impressed with your professionalism.
4. Take your time. Think before you answer. Do not allow yourself to be stampeded.
5. If you do not know, be honest and say so.
6. When you have answered the question, stop. Do not volunteer information. Keep your answers short and to the point.
7. The fair and honest witness will impress the judge.

**Re-examination**

Once the opposing counsel has completed cross-examination, your own barrister will have an opportunity to further question you about matters which have arisen out of cross-examination. This is usually to clarify points.

The judge may also wish to ask questions.

## Conclusion

**Some specific and general points**

Once you are sworn in to give evidence, you cannot talk to anyone about your evidence to the court and this includes your own barrister, e.g. during a lunch-break.

1. Consultation is highly advisable to highlight issues and relevant facts. If there are any points which you wish to raise with your client's representative, take a note of these in advance and deal with them at consultation.
2. Know where the court is and turn up in plenty of time.
3. Let your client's representative know that you have arrived as soon as possible

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This paper is based on a presentation given at the autumn scientific meeting of the Fish Veterinary Society in Edinburgh on 14 November 2002. It was submitted for publication on 13 January 2004.

PROCEEDINGS

*Fish Veterinary Journal (2005) (8) 101–106*

# Ulcer disease in common carp fisheries in the UK

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CEFAS, Weymouth Laboratory, Barrack Rd, Weymouth, Dorset DT4 8UB, UK (© British Crown copyright 2005)

*Abstract*

*Ulcer disease (or carp erythrodermatitis) associated with atypical* Aeromonas salmonicida *was diagnosed in two common carp fisheries in England. Managers from these two fisheries reported mortality rates over 30%. Clinical signs in fish included severe dropsy, exophthalmia, and varying sizes of skin ulcers. Fish were not systemically infected with atypical* A. salmonicida*; however, the bacteria was isolated from ulcers. Mortality was suspected to be due to osmoregulatory failure. Treatment options for this condition in fisheries are limited due to the extensive nature of these operations and, therefore, managers may want to focus on prevention strategies. Preventing stress on fish and improving water quality should help reduce the risk of this condition.*

## Case report

Ulcer disease, a bacterial infection of the skin, also referred to as erythrodermatitis was diagnosed in two independent carp fisheries in the UK during the summer of 2003. The managers from these two fisheries reported over 30% mortality in their fish. Affected fish included mirror carp (*Cyprinus carpio*) and hybrid carp, which was thought to be a cross between crucian carp (*Carassius carassius*) and common carp.

Clinical signs associated with this disease include varying sizes of skin ulcers, exophthalmia and ascites (Figure 1). In severe cases, scales protrude to give the fish a pinecone appearance, often referred to as dropsy. Although several types of bacteria may be isolated from the skin ulcers of affected fish, certain strains of *Aeromonas salmonicida* are believed to play the most significant role in the disease (Elliot and Shotts, 1980; Robertson and Austin, 1994; Noga, 2000). In an unrelated investigation of ulcer disease in goldfish (*Carassius auratus*), we cultured (and confirmed by 16S rRNA sequencing) atypical *A. salmonicida* from the lesions of two fish which had early stages

FIG 1: Common carp with 'ulcer disease' caused by atypical *Aeromonas salmonicida*.

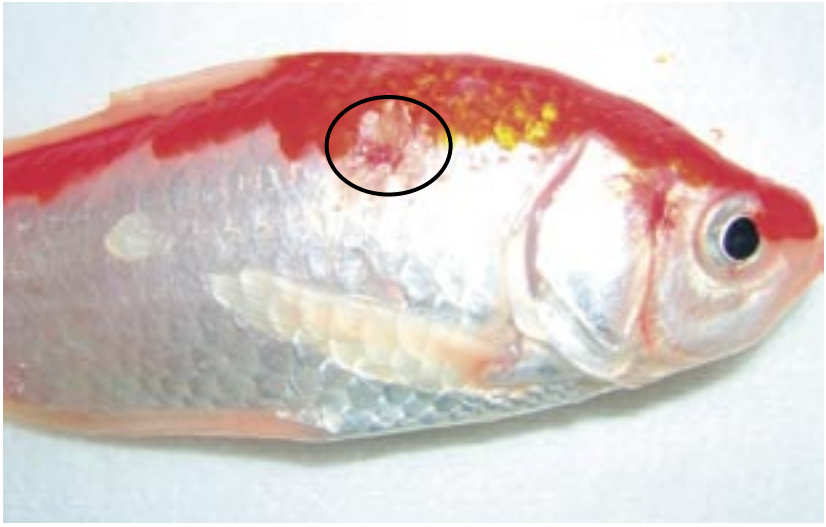

FIG 2: Early stage of ulcer disease in goldfish from an unrelated case. Circled area demarcates area of increased mucus production from which *A. salmonicida* was cultured.

of ulcer disease (Figure 2). No other bacteria were detected in these two fish; however, a bacterial culture from the lesions of a fish with advanced stages of ulcer disease, in the same tank as the fish with early signs of the condition, revealed mixed bacterial growth with *Enterobacteria* species and *Aeromonas hydrophila* predominating. These findings are consistent with published observations that initial ulcerative lesions are due to atypical *A. salmonicida* and other bacteria are secondary pathogens.

The *A. salmonicida* associated with ulcer disease is classified as an atypical subspecies (Hoole *et al*., 2001), and in carp it is generally not found in organs other than the skin (Wiklund and Dalsgaard, 1998; Noga, 2000). Mortality in affected fish is thought to occur because of osmoregulatory failure (Noga, 2000).

The clinical signs observed in the carp, which died in the two fisheries, were not unique to one fish pathogen. In India and other parts of the world *A. hydrophila* has been associated with dropsy and ulcerative skin lesions in *Cirrhinus mrigala, Catla catla, and Labeo rohita* (Shome *et al*., 1999). Fish infected with spring viremia of carp virus (SVCV) may also have similar clinical signs (Hoole *et al*., 2001). In the UK where SVCV is not common, the most probable cause of these signs is bacterial infections. Preliminary diagnosis of bacterial skin infections can be based on Gram stains of skin scrapings. Confirmation of the pathogen, however, requires culture and identification, which can be problematic if the causative agent is atypical *Aeromonas salmonicida*. Unlike many other bacteria isolated from the skin, this particular pathogen is difficult to grow and identify in the laboratory (Noga, 2000). In our investigation we were only able to isolate the bacteria from the fish when skin swabs were taken from the active edges of ulcers and plated on trypticase soy agar with 1% Coomassie brilliant blue (CBB R-250). Cultures were incubated at 22°C for 5 days.

Early identification of ulcer disease may facilitate a response to treatment. As the cause of mortality in carp with ulcer disease, in at least some cases, is believed to be osmoregulatory failure, salt has been suggested as one of the treatment options (Noga, 2000). Another treatment that may be considered is the use of antibiotics (Noga, 2000). However, when controlling the disease in a carp fishery the latter may be logistically difficult to administer properly. For advice on prevention and control of 'ulcer disease' caused by atypical *A. salmonicida* consult a veterinarian who specializes in aquatic animals.

Because of the difficulty in treating fish in fisheries it is important to prevent ulcer disease. Preventing exposure to the pathogen in a fishery environment may be difficult as *Aeromonas spp*. are commonly found in the aquatic environment; however, reducing exposure to factors that predispose fish to disease may help reduce the likelihood and/or severity of ulcer disease. Transmission trials, where affected fish from one of the fisheries with ulcer disease were transported back to the laboratory and cohabited with naïve carp, suggested that other factors besides the presence of the bacteria may play a role in the severity of this disease. In our trial only 2 of the 40 naïve fish developed small ulcers, and none of the fish had ascites or died. In fact, some of the original affected fish recovered once they were in our facility. At the end of the trial, atypical *A. salmonicida* was confirmed on the skin of 4 of 12 original fish, and in 1 of the 2 naïve fish with ulcers. Although it was not possible to confirm why the severity of the disease was less in a laboratory setting than the natural environment several factors such as water quality, stocking density, different feed rations, lack of natural predators, and lack of water temperature fluctuations may have played a role. In both outbreaks reported to CEFAS in 2003, there was the possibility of transport stress prior to the disease occurring. Fisheries managers should minimize the level of stress on the their fish as much as possible.

It is also possible there are susceptibility differences between fish strains. One research group determined carp from Hungary were more resistant to a specific isolate of atypical *A. salmonicida* than a Polish strain of carp (Houghton *et al*., 1991). Similar differences may occur between carp strains found in the UK.

In the late 1980's there was some research into the development of a vaccine against carp erythrodermatitis (Evenberg *et al*., 1988). In a laboratory-based challenge the vaccine injected intramuscularly appeared to offer some protection against the disease. More recently there has been an experimental (oral and immersion) vaccine used in ornamental fish to prevent ulcer disease (Schering-Plough Aquaculture). This vaccine, which contains both *A. hydrophila* and *A. salmonicida* antigens has had promising results under experimental conditions. (Personal communication Patrick Smith, Schering -Plough Aquaculture).

One aspect of the disease that is unknown and important in developing effective prevention strategies is whether fish can become carriers of atypical *A. salmonicida* and shed the infectious agent when they are stressed. This phe-

nomenon has been reported with *A. salmonicida* in salmonids (Noga, 2000); however, this state is generally associated with systemic bacterial infections, rather than localized infections as occurs in carp infected with the atypical *A. salmonicida*. Understanding this aspect of the epidemiology of ulcer disease may assist in providing better prevention strategies for the disease.

## Acknowledgment

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PROCEEDINGS

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# Investigation of a wild fish kill on the River Tyne

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*Abstract*

*A wild adult Atlantic salmon and sea trout fish kill occurred on the River Tyne in the summer of 2003. Similar mortalities have occurred in past summers on this river. The mortality in 2003 was attributed to* Vibrio anguillarum *and it is suggested that infection with this pathogen was most likely exacerbated by the low water flow, which impaired fish movement upstream and lead to large aggregations of fish, and high water temperatures, which increased the replication rate of the bacteria and stressed the fish. Both of these environmental conditions would have favoured fish-to-fish transmission of the bacteria, and increased the mortality rate associated with the pathogen.*

## History

In the summer of 2003 there were reports of adult Atlantic salmon (*Salmo salar*) and, to a lesser extent sea trout (*Salmo trutta*), dying along a 4 km section of the River Tyne between Wylam, Northumberland, and Newburn, Tyne and Wear, England. This stretch of the river is at the upper limit of tidal influence and, therefore, the water level fluctuates with the tides, but remains fresh. The water level in the summer and autumn of 2003 was very low and temperatures in July and August were high (range from 17 to 24°C). At the time of sampling in September, the water temperature was between 17 and 18°C and fish were aggregating below the weir in Wylam due to the low water levels (Figure 1).

Since the start of the mortality in July, the Environment Agency removed dead fish on a bi-weekly basis from the affected area. In the summer of 2003 it was estimated that at least 25% (1000/4000) of the fish that entered the river died (Norton, 2003a; Norton, 2003b). The numbers of dead fish appeared to be decreasing by the end of September 2003.

On September 10th a sample of three fish was collected and processed at the CEFAS Weymouth Laboratory in Weymouth, England. All fish sampled had

FIG 1: Weir on the River Tyne at Wylam.

been dead for at least a day. A subsequent sample of four fish was collected on September 17th to confirm preliminary findings. Two of these fish were dead; one was estimated to have died a few hours prior to sampling and the other had been dead for at least 24 hours.

## Diagnostic work-up

A routine diagnostic work-up was conducted on the fish sampled. This consisted of Gram stain of liver and kidney imprints, Diff Quick™ stain of blood smears, and bacteriological culture of kidney swabs on tryptone

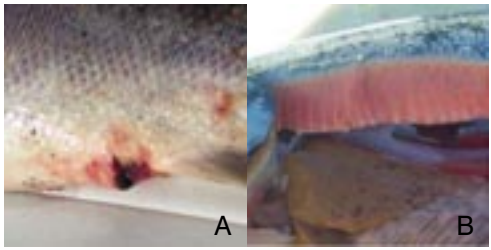


FIG 2: A) Reddened vent and B) haemorrhages in the liver of the dead Atlantic salmon sampled in 2003 on the River Tyne. (photo B is courtesy of Cilka Latrace)

soya agar (TSA) incubated at 22°C for 5 days. Confirmation of *Listonella anguillarum* (formally known as *Vibrio anguillarum*) (Austin and Austin, 1993) was done using primary diagnostic tests (colony morphology, motility, Gram stain, catalase and oxidase test) and secondary tests, including API 20 E (Biomerieux, Marcy L'Etoile, France) and a rapid agglutination test for *L. anguillarum* (Mono-Va Bionor, Skien, Norway).

Virology samples were collected from fish sampled on September 10th. Viscera and gills from these fish were inoculated on to bluegill fry cells (BF-2) with and without IPN antisera, chinook salmon embryo (CHSE-214) and *Epithelioma Papulosum Cyprini* (EPC) cell lines. The procedure used for virology is described in the *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2003).

Representative samples of visceral organs, kidney, gills and skin from all fish that were less than 24 hours old were fixed in 10% neutral buffered formaldehyde and processed using standard histological procedures and Haematoxylin and Eosin (H&E) staining.

## Findings

Most fish had reddened and hemorrhagic vents (Figure 2). Some fish also had reddened areas on the skin with some scale loss. Skin lesions occurred mostly around the base of the tails, and, internally, fish had areas of haemorrhage in the liver (Figure 2). The two females examined on September 17th had low body fat stores. Gills appeared normal on physical examination.

All virology tests were negative for the presence of virus. No blood parasites were detected; however, bacteria were present in all blood smears. Gram stain of the tissue imprints from the first three fish sampled had a moderate to heavy presence of short Gram-negative rods. Very few bacteria were present on the tissue imprints from the second group of fish sampled.

Bacterial cultures obtained from the kidneys of 3 of the 6 fish tested yielded significant pure growth of *Listonella anguillarum*. Another fish tested yielded a mixed bacterial growth with *L. anguillarum* predominating. One fish yielded no bacterial growth. A further fish was not tested due to advanced autolysis.

Histological findings were consistent with the presence of a bacterial infection. Kidneys had focal areas of tissue necrosis; livers and intestinal tracts had degenerative changes, some of which may have been due to post mortem

autolysis; spleens were congested; gill structure appeared normal, however, *Ichthyophthirius multifiliis* parasites were present in low numbers. There was no evidence of other infectious agents causing significant pathology.

## Conclusions

The cause of the Atlantic salmon mortality on the River Tyne was attributed to *L. anguillarum*. *Vibrio* species are known to exist in the aquatic marine environment and some species are more pathogenic to fish than others (Austin and Austin, 1993). *Listonella anguillarum* is known to be pathogenic to salmonid species (Kent and Poppe, 1998; Spanggaard *et al*., 2000). Its pathogenicity is determined by the presence or absence of several virulence factors (Pedersen and Larsen, 1998).

Environmental stressors may also influence the severity of disease associated with *L. anguillarum* by compromising the immune system of fish (Jacobson *et al*., 2003). In particular, infections are most problematic when fish are held under stressful conditions and high water temperatures (Kent and Poppe, 1998). Several studies have demonstrated fish exposed to stressors, such as various types of pollutants, suffer higher losses when exposed to *L. anguillarum* (Arkoosh *et al*., 2001; Jacobson *et al*., 2003).

Mortalities of Atlantic salmon have been reported in the River Tyne for several years in the late summer and early autumn. In 1995, CEFAS scientists isolated a *Vibrio* sp. in a number of fish from this river during a fish kill. In other years fish have been examined by the staff of the National Fisheries Laboratory (Environment Agency) in Brampton, England. They have also reported a variety of bacteria in dead fish, including a *Vibrio* sp. Although it was not possible to determine whether the bacteria isolated during this investigation had any or all of the virulence factors associated with highly pathogenic strains of *L. anguillarum,* it is suspected that this was the causative agent for the mortality in Atlantic salmon in this river. The bacteria were isolated in significant quantities from almost all fish sampled, and dead fish had clinical signs consistent with vibriosis. Environmental conditions in the river, in particular low water flows, which prevented fish from dispersing, as well as high water temperatures in July and August may have exacerbated the mortalities caused by this pathogen. In our opinion, these conditions would have increased the numbers of bacteria present in the water; caused the aggregation of fish in pools, which, in turn, would have increased the probability of fish to fish contact and spread of the pathogen. Environmental

parameters such as oxygen and temperature were, during some periods of the day, at the upper end of the tolerance range for salmonids (Piper *et al*., 1982). This may also have increased the physiological stress on the fish.

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*Fish Veterinary Journal (2005) (8) 114–115*

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Please use Block Capitals

Name: _____

Organisation: _____

Address: _____

_____

_____

Telephone: _____ Fax:_____

*Please return this form with full payment to:*

J. McArdle
Fish Veterinary Journal
119, Park Drive Avenue
Castleknock
Dublin 15, IRELAND

## MEMBERSHIP APPLICATION

**Eligibility**
Membership of the Fish Veterinary Society is open to all members of the Royal College of Veterinary Surgeons, to those on the Supplementary Veterinary Register and to students studying for a degree entitling them to membership of the RCVS. The Society will also consider applications from overseas veterinarians and those with an appropriate interest / degree as set out in the Constitution of the Society (available on request from Treasurer).

I wish to become a member of the Fish Veterinary society, subject to the conditions governing the same as set out in the Constitution of the Society.

I agree to pay my annual subscription in advance on 1st January each year, and if at any time I wish to resign from membership, undertake to send my resignation to the Honorary Treasurer by 1st December.

**Name** :

**Address** :

**Telephone** : **Fax**:

**e-mail** :

**University** :

**Degrees** :

**Date of graduation** :

**Fees** **: £50 per annum** (January to December)
(There is no charge to veterinary undergraduates)

*The sum of £50 is enclosed for full enrolment into the Fish Veterinary Society and membership for the current year. Future payments will be made by Standing Order each year in January (mandates available from Honorary Treasurer)

*I am a veterinary undergraduate and wish to become an associate member of the Fish Veterinary Society and I am due to graduate in_____(year)

**delete as appropriate*

Signature :

## MEMBERSHIP DATABASE

To help the Society provide a better service to its members we would be grateful if you could complete the following questionnaire by indicating your area of special interest.

**Species of interest:**

| | | | |
|---|---|---|---|
| Salmon | ❒ | Trout | ❒ |
| Flatfish | ❒ | Shellfish | ❒ |
| Ornamental | ❒ | Other (please specify)… | |

**Areas of interest:**

| | | | |
|---|---|---|---|
| Pathology | ❒ | *Histopathology* | ❒ |
| *Bacteriology* | ❒ | *Parasitology* | ❒ |
| *Mycology* | ❒ | *Virology* | ❒ |
| Diagnostics | ❒ | Genetics | ❒ |
| Immunology | ❒ | Legislation | ❒ |
| Management | ❒ | Nutrition | ❒ |
| Pollution | ❒ | Reproduction | ❒ |
| Surgery | ❒ | Therapeutics | ❒ |
| Water Quality | ❒ | Welfare | ❒ |
| Other (please specify)… | | | |

**Other Membership:**

| | |
|---|---|
| British Veterinary Association | ❒ |
| British Small Animal Veterinary Association | ❒ |
| European Association of Fish Pathologists | ❒ |
| British Trout Association | ❒ |
| Scottish Salmon Growers Association | ❒ |
| Institute of Fisheries Management | ❒ |

Return to: Chris Walster MRCVS
Island veterinary Clinic
132 Lichfield Road
Stafford
Staffordshire ST17 4LE
telephone 01785 258411
fax 01785 258416
email cwalster@aol.com