IMPACT OF ANTIBACTERIAL DRUGS ON THE HEALTH OF EUROPEAN EEL

Promotor:	Dr. E.A. Huisman, hoogleraar in de Visteelt en Visserij
Co-promotoren:	Dr. J.H. Boon, universitair hoofddocent in de Visgezondheid
	Dr. J.H.W.M. Rombout, universitair hoofddocent in de Zoölogische Celbiologie

IMPACT OF ANTIBACTERIAL DRUGS ON THE HEALTH OF EUROPEAN EEL

M.H.T. VAN DER HEIJDEN

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Abstract

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Application of antibacterial drugs plays an important role in the control of bacterial diseases in intensive fish culture. However, since the scientific basis for the use of these drugs is yet inadequate, there is a growing concern about the safety of fish medication for the consumer, the environment and the target animals. This led to the research presented in this thesis, which is focused on the influence of the antibacterial drug flumequine on the defence system of European eel (*Anguilla anguilla* L., 1758). Eel appeared to be an interesting model because the initial, pharmacokinetic investigations demonstrated that flumequine is very slowly eliminated from both plasma and tissues in this species. Treatment of eels with flumequine resulted in an enhanced proliferation of (probably surface immunoglobulin negative) lymphoid cells as assessed by means of an *in vivo* lymphocyte stimulation assay and monoclonal antibodies to eel immunoglobulin, respectively. The effect of the drug on the integral functioning of the defence system was subsequently examined by using a challenge with the parasitic nematode *Anguillicola crassus*. The resulting parasite recoveries pointed to an improved protection in flumequine-treated eels, which seemed to be related to a modulation of the cellular response.

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Stellingen

1. Voor het aantonen van antilichaam responsen tegen *Anguillicola crassus* bij aal zijn frequente infecties met infectieuze larven van deze parasiet noodzakelijk. *Dit proefschrift*

2. Het immunostimulerende effect van flumequine mag niet bepalend zijn voor het gebruik hiervan in de aalteelt. *Dit proefschrift*

3. Ook voor farmacotherapie geldt: de ene vis is de andere niet. *Dit proefschrift*

4. De relevantie van *in vitro* vastgestelde immunotoxiciteit dient gevalideerd te worden met behulp van *in vivo* testen.

5. Gezien de specifieke complicaties bij de diagnose en behandeling van visziekten is ziektepreventie in de visteelt nog belangrijker dan in andere vormen van dierhouderij.

6. Onderzoek naar de invloed van geneesmiddelen op de afweer leidt tot efficiëntere ziektetherapieën.

7. De beperkte toegankelijkheid van de Japanse literatuur werkt vertragend op het Europese onderzoek aan aal.

8. Bij een discussie over het al dan niet opnemen van de evolutietheorie in het eindexamen voor middelbare scholieren dient de nadruk te liggen op de bruikbaarheid van deze theorie voor de verklaring van biologische verschijnselen.

9. Door het ontbreken van visziekte-onderwijs in het curriculum van aanstaande dierenartsen is het ongeloofwaardig dat het recht tot het voorschrijven van geneesmiddelen voor vissen aan dierenartsen is voorbehouden.

10. Gezien de bezuinigingen op de budgetten in het wetenschappelijk onderwijs is de winststijging van de KLM niet veroorzaakt door een toename in het bezoek van internationale congressen door Nederlandse universitaire medewerkers.

11. Bij het uitblijven van adequate maatregelen ten aanzien van de controle op residuen van geneesmiddelen in consumptievis zou deze op termijn nog slechts op recept verkrijgbaar moeten zijn.

Stellingen behorend bij het proefschrift "Impact of antibacterial drugs on the health of European eel" van M.H.T. van der Heijden. Wageningen, 1 april 1996.

Table of contents

Chapter 1

General introduction

Partly based on: Van der Heijden, M.H.T., van Muiswinkel, W.B., Grondel, J.L., Boon, J.H., 1992. Immunomodulating effects of antibiotics. In: C. Michel and D.J. Alderman (Editors), Chemotherapy in Aquaculture: From Theory to Reality. Office International des Epizooties, Paris, pp. 219-230.

Contents

Introduction

The global increase in fish culture production is a result of both aquaculture area expansion and production intensification (Huisman *et al.*, 1993; Anonymous, 1994). High densities of fish stocks in intensive aquaculture systems are accompanied by an increased prevalence of infectious diseases as a result of a higher infection pressure and decreased resistance of the fish (Roberts, 1989). Economical losses related to diseases are caused by reduced growth and morbidity and mortality among the animals. It is attempted to minimize these losses by optimization of culture conditions through zootechnical and hygienic measures. Also, vaccination is a promising approach, but, for the time being, effective vaccines are only available for a limited range of fish pathogens (Ellis, 1988). However, in view of the limitations of the control methods mentioned, chemotherapy is still considered the most effective weapon against infectious diseases in aquaculture (Gratzek, 1983; Stuart, 1983). In as far as bacterial infections are concerned, a variety of antibacterial drugs is used, curatively as well as prophylactically (Austin, 1984).

Although antibacterial drugs are known to be of remarkable value for the control of bacterial fish diseases (Gratzek, 1983; Ellis, 1988), a growing interest in the possible hazards due to the use of these drugs in aquaculture can be observed. Bernoth (1991) paid attention to the risks concerning public health, i.e. residues of drugs in fish destined for human consumption, development of resistance in human pathogenic bacteria and the direct toxic effects on humans from handling the drugs. Furthermore, treatment of fish causes pollution of the environment, which may result in a negative selective influence on the growth of the normal bacterial fauna and an increase of resistant pathogens (Austin, 1985; Lunestad, 1992; Samuelsen, 1993). Last but not least, antibacterial drugs can exhibit undesired side effects on the health of the target animals. Among these, gastro-intestinal disturbances, damage to organs such as liver and kidney, and superinfections with viruses, resistant bacteria, fungi and parasites have been described in clinical reports (Garrod *et al.*, 1973). Prolonged exposure to higher concentrations of a drug, as may be expected in liver and kidney, seems to affect the occurrence of lesions. Moreover, especially rapidly dividing cells like intestinal epithelium and activated lymphocytes appear to be prone to toxic effects of drugs (Claassen, 1991).

During the last decades, studies have been performed on the interaction between

antibacterial drugs and the immune system in mammals, birds and fish (Grondel and van Muiswinkel, 1986). Some antibacterial drugs have shown to interfere with the immunological defence mechanisms. This interference may result in immunostimulation or immunosuppression. The latter situation is greatly undesirable, because an optimal immune function is important for both the final elimination of the pathogens that gave rise to the therapy and the resistance against new infections. Immunostimulation, on the other hand, fits within the purpose of an antibacterial treatment, because it may enhance defusion and elimination of the pathogen.

A widespread use of antibacterial drugs in intensive fish culture systems, in combination with their potential immunomodulating properties and the associated influence on the health of treated animals, gave cause to the research presented in the current thesis. This chapter is a general introduction to the basic subjects raised in the thesis. First, some aspects of the biology and culture of European eel (*Anguilla anguilla* L., 1758), the central species in the experiments described, will come up. Secondly, attention will be paid to the antibacterial drugs and the pharmacokinetics of such drugs in fish. Subsequently, the immune system of teleost fish will be briefly discussed, followed by some comments on immunomodulation and the assays to detect it. Finally, the aim and outline of the thesis will be described.

European eel (*Anguilla anguilla* L., 1758)

Biology of the European eel

Since ancient times many people look upon eels as mysterious, snake-like creatures because of their peculiar habits and unusual body shape. Numerous scientific investigations on eel biology (Liewes, 1981; Tesch, 1983; Deelder, 1984; Tesch, 1991) have contributed to an increased knowledge and understanding of these fish. Nevertheless, important aspects of their live and functioning still have to be elucidated.

The European eel (*Anguilla anguilla* L., 1758), which is among the best studied eels, is a teleost fish classified in the genus *Anguilla* (Shaw, 1803) together with 15 other species and 3 subspecies (Tesch, 1991). The (sub)species within this genus show a high degree of mutual resemblance with notable external characteristics such as the very elongated body, dorsal and anal fins confluent with a rudimentary caudal fin and

absence of pelvic fins. *Anguilla* spp. are usually distinguished by their geographical distribution, possibly in combination with their number of vertebrae or myomeres. Other common determination features are the colour of the skin, length of the dorsal fin, and construction of the jaws, including implant of the teeth. However, the latter features are age-dependent and can therefore only be used to discern individuals of approximately the same age.

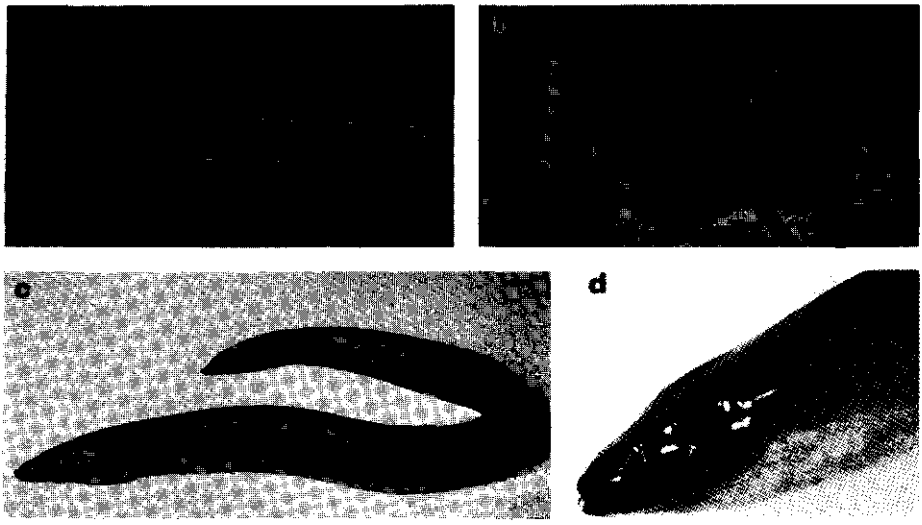

Figure 1. Different developmental stages of the European eel (*Anguilla anguilla* L., 1758). (a) Leptocephalus (from Tesch, 1983); (b) glass eels (from Usui, 1991); (c) yellow eel; (d) silver eel (from Tesch, 1991).

Wild European eel is supposed to spawn in the Sargasso Sea (26° N.lat. and 60° W.long.) at great depth with a peak spawning activity in March. This hypothesis is based on a spatial and temporal gradient in the size of caught eel larvae, the so-called **leptocephali** (Figure 1a). The exact spawning site is still unknown as sexually mature eels or fertilized eggs have never been found. During about 1 to 2.5 years the leptocephali migrate both actively and passively in the direction of the European and North African coast and finally metamorphose into the transparent **glass eels** (Figure 1b). Except for the pigmentation, the eels now have almost attained their final

appearance. The arrival of glass eels in estuaries and coastal waters depends on the distance of the particular coastal region from the spawning area. Once in fresh water, upstream swimming and feeding starts and a black pigmentation develops; these young eels are now called **elvers**. As pigmentation progresses, their colour gradually turns more or less yellowish and the **yellow eel** stage (Figure 1c) is reached. Yellow eels may stay in fresh water for many years. During this period the sexual differentiation of the animals starts, but they do not attain sexual maturity. After at least 4 to 5 years, depending on sex and associated size, the eels gradually transform into **silver eels** (Figure 1d). In this stage the fish morphologically and physiologically adapts to the upcoming return to the deep-sea spawning grounds. Clear external changes are development of a ventrally spreading silvery glow on the flanks and enlargement of the eyes. Besides, endocrine changes, growth of the gonads, migratory restlessness and cessation of feeding together with degeneration of the digestive tract have been reported. Eels are assumed to return to their parent spawning area, to spawn once and die.

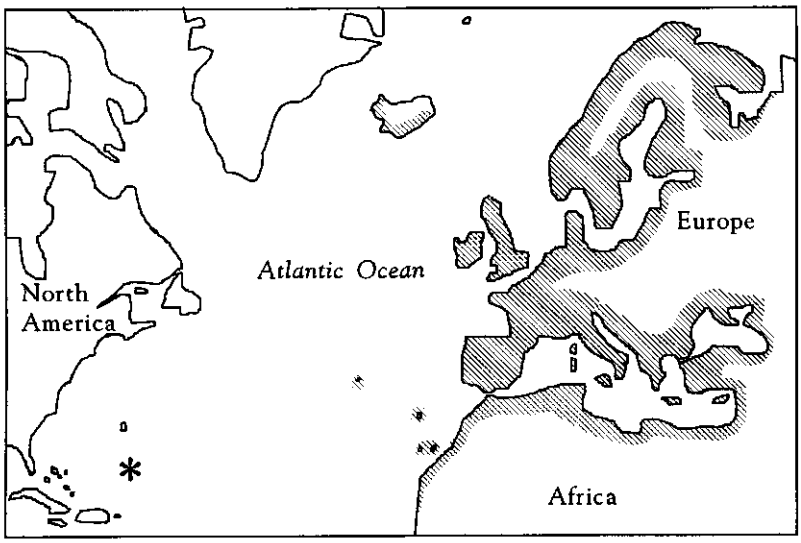

Figure 2. Map showing the natural geographic distribution of the European eel (*Anguilla anguilla* L., 1758; indicated by the shaded area) and the location of the supposed spawning grounds in the Sargasso Sea (indicated by an asterisk; after Tesch, 1983).

The natural geographic distribution of the inland stages ranges from the North Cape to the Atlantic coast of Morocco and the Canary Islands, and from the Black Sea to Iceland and the Azores (Figure 2). The distribution area is limited by the need to ascend from the sea. Thus, drainage areas directly connected to the Atlantic Ocean are favoured. Most conspicuous are the broad tolerance ranges with respect to environmental circumstances. Eels inhabit the cold northern European waters, whereas temperatures of up to 36° C are survived for at least several days (Schippers *et al.*, 1991). As eels are euryhaline fish, they can be transferred smoothly from fresh to salt water and vice versa. If protected from drying out, they even survive outside the water. The eels live purely solitary and, dependent on their size, mainly predate on fish, oligochaetes or arthropods. However, as a result of their high fat content, they endure starvation for a considerable time.

Willemse (1979) studied the internal morphology of the European eel. Although usually in a more elongate shape, the regular organs of teleost fish are present (Figure 3), including the major lymphoid organs such as head and trunk kidney, spleen and thymus. Remarkable is the presence of a Y-shaped stomach, a large gallbladder and a prominent urinary bladder as well as the location of the kidney. The greater part of the excretory kidney (the trunk kidney) is situated postanally. At the rostral end the trunk kidney gradually changes into long, thin strands on both sides of the dorsal aorta. Histological investigations at our laboratory showed that the paired kidney strands consisted almost exclusively of lympho-myeloid tissue (not shown), suggesting homology with the much more rounded and rostrally situated head kidney (pronephros) in other teleosts. The excretory kidney of fresh water adapted eel contained, next to interrenal haematopoietic tissue, relatively few glomeruli, with long tubules compared to stenohaline fresh water fish.

Culture of European eel

According to the latest figures, the world aquaculture production of *Anguilla* spp. is about 10^5 metric tonnes (MT) yearly (Anonymous, 1994). The major part is produced in Japan and Taiwan and consists mainly of Japanese eel (*Anguilla japonica* Temminck and Schlegel, 1846). Culture of European eel is concentrated in Europe and totals about $7\text{-}8 \cdot 10^3$ MT per annum. Eel culture in Japan and Europe was reviewed by Heinsbroek (1991).

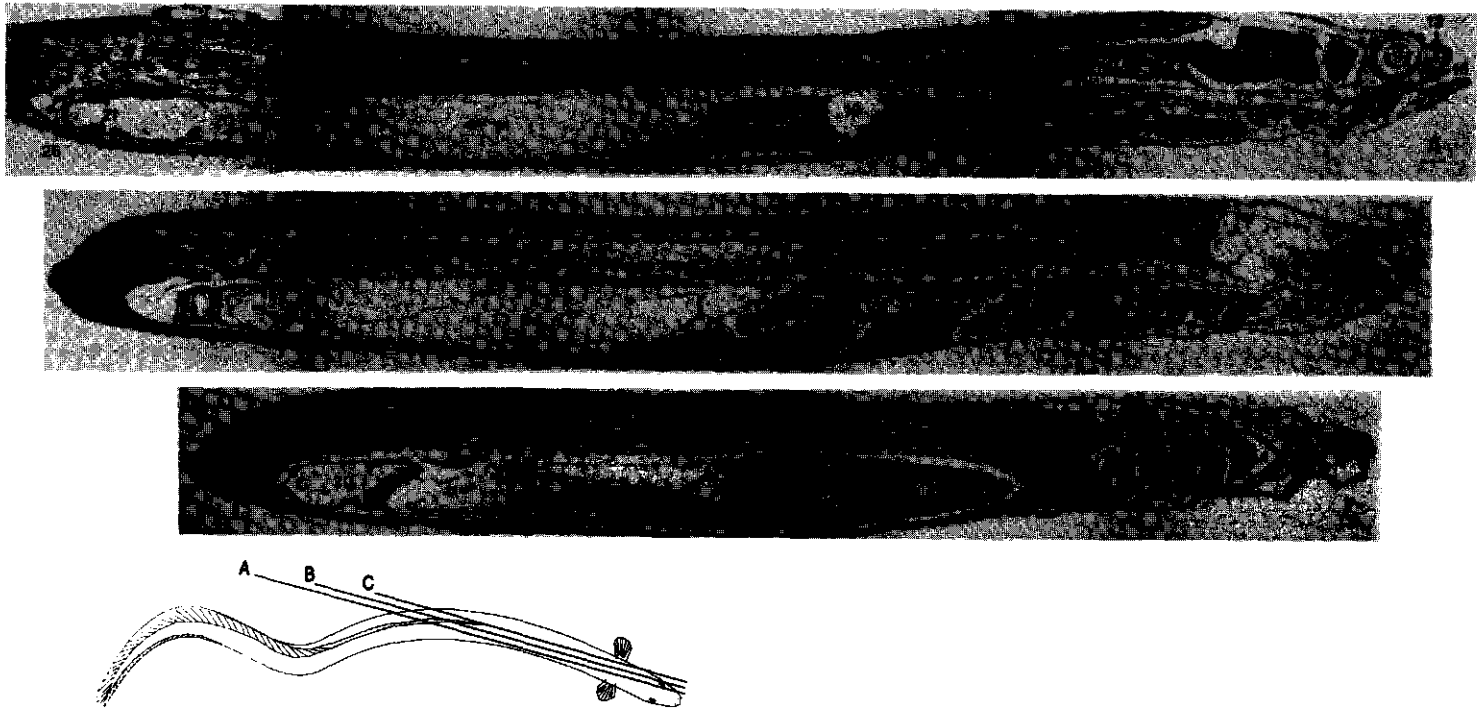

Figure 3. **Sagittal sections of small (8-10 cm) yellow eel specimens.** Fish were fixed in Bouin's solution, embedded in paraffin wax and sectioned at 5 μm. Sections were stained according to the Crossmon method. Scale bar = 0.5 mm.

1	Anterior intestine	8	Oesophagus	15	Mouth	22	Spinal cord
2	Atrium	9	Eye	16	Vertebral column	23	Stomach
3	Brain	10	Gallbladder	17	Otolith chambers	24	Thymus
4	Buccal cavity	11	Swimbladder	18	Pharynx	25	Trunk kidney
5	Bulbus arteriosus	12	Gills	19	Posterior cardinal vein	26	Urinary bladder
6	Dorsal aorta	13	Lateral line canal	20	Posterior intestine	27	Ventricle
7	Dorsal fin	14	Liver	21	Rectum		

After the first attempts to raise eels under controlled circumstances were made in Europe, commercial eel culture actually started in Japan at the end of the last century. The initially primitive culture techniques have gradually been replaced by intensive culture methods. Nowadays, a production of about $40 \cdot 10^3$ MT of eels per year is realized only in Japan (Anonymous, 1994). The majority of the eels (80-90%) is raised in greenhouse ponds at densities of 5-20 $kg \cdot m^{-2}$ and temperatures of 22-28°C. Water quality is maintained by aeration and partial water exchange with fresh well water. Accordingly, stocked glass eels reach the marketable size of 150-200 g in about 1-1.5 year.

Eel culture in Europe has its origin in the Mediterranean area, notably Italy. This country is still the most important producer of European eel. Their culture is based on low-intensity aquaculture in brackish water lagoons. However, since the sixties a shift towards more intensive freshwater culture, using adapted Japanese farming techniques, is observed.

The attractive market prices of eels, caused by declining catches (Tesch, 1991; Buijse *et al.*, 1992) in combination with a high demand, have also created a strong interest in eel culture in north-western Europe. First, culture in heated effluents was attempted, but, due to disappointing results, this form of eel culture is declining. About two decades ago, the development of water recirculation systems for intensive culture of warm water species gave the opportunity for a new type of eel culture, which presently covers circa 30% of the eel culture production in Europe. These closed systems are stocked with glass eels or farm-raised elvers. The animals are frequently graded (every 1-4 month) and as their size increases they are transferred to larger tanks and stocked at higher densities (up to 75 $kg \cdot m^{-2}$). The water temperature is kept at 20-25°C and feeding is almost exclusively done with compound feeds, either in pelleted or in paste form. Under these circumstances it takes about 1.5-2 years until the fish can be harvested (150-200 g), which is somewhat longer than the time needed to raise Japanese eels. This discrepancy is attributed to a lower growth performance of the European species (Tesch, 1983; Heinsbroek, 1991).

In spite of partly successful attempts for artificial reproduction of eels (Yamauchi *et al.*, 1976; Boëtius and Boëtius, 1980), production of glass eels in captivity is still not possible. Therefore, farming of eels totally depends on collection of wild, non-domesticated stocking material. Especially in the initial culture phases, growth and survival are severely affected by non-acceptance of feed and infectious diseases

(Heinsbroek, 1989). This may be connected with possible import of pathogens (Tesch, 1991) and stress associated with the culture conditions (Peters *et al.*, 1980), the latter also being a major cause for disease problems in the more advanced culture phases. Numerous pathogens have thus far been isolated from cultured eels (Reichenbach-Klinke, 1980), however only few appear to be of importance with regard to clinical diseases. Next to some ectoparasitic infections caused by e.g. *Dactylogyrus* spp., *Pseudodactylogyrus* spp. and *Trichodina* spp. (Heinsbroek, 1991), bacterial diseases, which often start as opportunistic infections, may become very serious (Tesch, 1991). Koops and Kuhlmann (1983) even claimed that antibacterial drugs are indispensable for successful production in intensive eel aquaculture.

Antibacterial drugs

Antibiotics are chemical substances produced by various species of micro-organisms that either suppress the growth of other micro-organisms (bacteriostatic) or even kill them (bactericidal). Those with selective toxicity towards bacteria without interfering with the biochemical pathways of eukaryotic cells are of potential value in the therapy of bacterial diseases. Originally, a distinction is made between chemical synthetic drugs with antibacterial properties (chemotherapeutic agents) and antibiotics. However, this difference has lost significance as most antibacterial drugs are largely or entirely synthetic at present. Therefore, and because it enables to refer to both types in one go, the term antibacterial drugs is preferred in this thesis.

The quantity of antibacterial drugs used in aquaculture on a worldwide basis is not available due to lack of control and record-keeping in most countries. In this respect Norway appears to apply the closest control because all drug use in veterinary medicine, including drugs used in aquaculture, is recorded by a few nationally authorized drug dispensing agencies. Figure 4 shows the total amount of antibacterial drugs used in Norwegian salmonid farming from 1980 until 1992 (Grave *et al.*, 1990; Martinsen *et al.*, 1993). Taking the stock expansion during this period into account, there is still an increase from about 200 $g \cdot MT^{-1}$ farmed fish in 1980 to about 600 $g \cdot MT^{-1}$ in 1987 (Grave *et al.*, 1990). Thereafter, these figures gradually returned to the amount reported for 1980 (Martinsen, 1993; Anonymous, 1994). The initial increase in drug use was caused by a high morbidity due to bacterial infections and development of drug

resistance. The following decrease was attributed to an effective vaccination programme against *Vibrio salmonicida* and a tendency to cyclic variation in the utilization of drugs (Grave *et al.*, 1990), but will also be affected by a shift to more potent drugs with lower doses per treatment. Notwithstanding the reduction in the last years, the total amount of antibacterial drugs needed to maintain the currently about 150×10^3 MT of salmonids in Norway is still considerable. Realizing that these data are difficult to extrapolate to other countries and fish species, they give at least an indication of the extent in which antibacterial drugs are used in intensive finfish production systems.

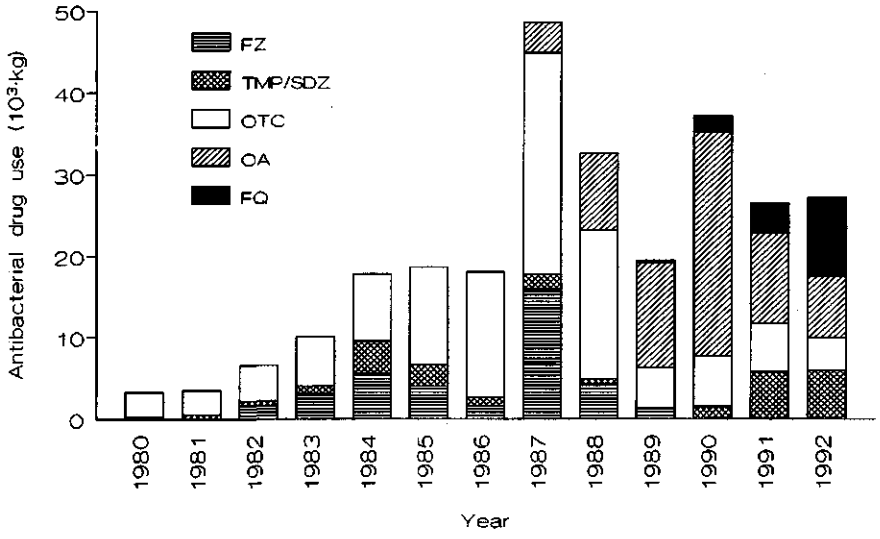

Figure 4. The use of antibacterial drugs in treatment of farmed salmonids in Norway in the period from 1980 to 1992. After data from Grave *et al.* (1990) and Martinsen (1993). Abbreviations: FZ, furazolidone; TMP/SDZ, trimethoprim/sulphadiazine; OTC, oxytetracycline; OA, oxolinic acid; FQ, flumequine.

The undesirably high amounts of antibacterial drugs used in intensive aquaculture may be illustrated by comparison with the prescription of such drugs in human and traditional veterinary (mainly cattle, swine and poultry) medicine. For the Dutch situation in 1990, Van den Bogaard *et al.* (1994) estimated that these antibacterial drug consumptions were 0.20 and 0.27 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively. Making this calculation

for the use of antibacterial drugs in Norwegian salmonid farming in the same year (assuming that the FAO production data for 1991 (Anonymous, 1994) represent the biomass of farmed salmonids in the preceding year) revealed 0.80 $mg \cdot kg^{-1} \cdot day^{-1}$. This is about 3 times higher than the amount used for intensively cultured traditional farm animals. Although this difference may partly be explained by the difficulty of administering a sufficient amount of a drug to the right fish, the figures suggest that a reduction of the prescribed quantity of drugs for fish may be possible. In this respect, progress may be achieved by the availability of drugs tailored for use in aquaculture, which will enable more effective treatment.

In The Netherlands, drugs specifically licensed for use in aquaculture are not available as yet. Nevertheless, drugs are regularly used for disease control at Dutch eel farms. Anthelmintics, mainly mebendazole, is used for the control of gill flukes (*Dactylogyrus* spp. and *Pseudodactylogyrus* spp.). Furthermore, bacterial diseases, mainly caused by the opportunistic, Gram-negative bacteria such as motile *Aeromonas* spp. and *Flexibacter* spp., are predominantly controlled with the antibacterial drugs flumequine, oxytetracycline and furazolidone. As the use of the latter drug is strongly discouraged in many countries and will probably be prohibited in the future, this thesis mainly deals with flumequine, while oxytetracycline, one of the most extensively studied drugs in fish, is sometimes used as reference.

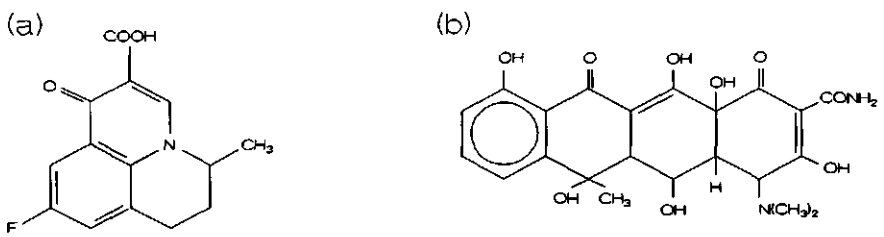

Figure 5. The chemical structure of (a) flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo[ij]quinolizine-2-carboxilic acid) and (b) oxytetracycline (4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide).

Flumequine

Flumequine (Figure 5) is a completely synthetic antibacterial drug and belongs to the so-called second generation quinolones. Like all quinolones flumequine was derived from nalidixic acid, the first clinically useful agent in this class of antibacterial drugs, which was originally isolated by Lesher *et al.* (1962). Flumequine was an important compound in the evolution of quinolones because of the introduction of a fluorine residue in the molecule, which enhanced and broadened the antibacterial activity compared to the previously available, non-fluorinated congeners (Andriole, 1988). It is a bactericidal agent, active against mainly Gram-negative bacteria (Smith and Lewin, 1988; Anonymous, 1989; Mevius, 1990). Table 1 presents minimum inhibitory concentrations (MICs) of flumequine for different fish pathogenic bacteria as described in literature (Ceschia *et al.*, 1987; Ledo *et al.*, 1987; Martinsen *et al.*, 1992; Palmer *et al.*, 1992).

The exact mode of action is still poorly understood (Crumplin, 1988; Diver, 1988). However, it is generally accepted that quinolones inhibit DNA synthesis by inhibition of the enzyme DNA-gyrase (Cozzarelli, 1980; Shen and Pernet, 1985; Wolfson and Hooper, 1985; Smith, 1986; Smith and Lewin, 1988; Drlica *et al.*, 1990; Vancutsem *et al.*, 1990). This is a bacterial type II topoisomerase and plays a crucial role in the unwinding, cutting and consecutive resealing of DNA. Inhibition of this enzyme results in filamentation of the bacterial cells, followed by vacuolization and ultimately death and lysis of the cells (Sonstein, 1990). Although it is known that active protein synthesis is required for these drugs to kill bacteria (Smith and Lewin, 1988), the actual lethal event is still unclear. Smith (1986) proposed that inhibition of the DNA resealing leads to exposed DNA nicks inducing exonuclease production, which in turn degrade the chromosomal DNA and consequently cause bacterial death. According to another hypothesis the bacteria are killed as a result of extensive induction of their own DNA repair system, and the ensuing gross alteration of the cell's biochemistry and morphology (Diver, 1988; Sonstein, 1990). Moreover, it has been suggested that quinolones have a second site of action, possibly the cell membrane, which contributes to cell death (Dougherty and Saukkonen, 1985; Chapman and Georgopapadakou, 1988).

Very unusually among antibacterial drugs, the quinolones are not affected by plasmid-mediated resistance (Courvalin, 1990). As a consequence of this the frequency of clinical resistance to quinolones is much less than that of other major groups of

antibacterial drugs (Smith and Lewin, 1988). Resistance to quinolones can only result from chromosomal mutations (non-transferable) that either diminish the affinity of the bacterial DNA-gyrase for the drug or decrease drug penetration into the cell (Barry, 1990; Lewin, 1992). Nevertheless, the increasing use of quinolones in aquaculture has led to development of resistant strains of fish pathogenic bacteria. Moreover, both mutual cross-resistance among quinolones and co-resistance to other structurally unrelated drugs have been reported in laboratory-derived mutants as well as in strains isolated from diseased fish (Tsoumas *et al.*, 1989; Barnes *et al.*, 1990; Lewin *et al.*, 1992; Martinsen *et al.*, 1992).

Table 1. Minimum inhibitory concentrations (MICs) of flumequine for different fish pathogenic bacteria

Bacterial species	MIC ($\mu g \cdot ml^{-1}$)	Reference
Gram-negative bacteria		
Aeromonas caviae	<0.075	Ledo *et al.* (1987)
Aeromonas hydrophila	<0.075	Ledo *et al.* (1987)
Aeromonas salmonicida	<0.01 - 0.78	Palmer *et al.* (1992)
Aeromonas sobria	<0.075	Ledo *et al.* (1987)
Edwardsiella tarda	<0.01 - 0.3	Ledo *et al.* (1987); Palmer *et al.* (1992)
*Listonella anguillarum**	<0.01 - 0.3	Ledo *et al.* (1987); Palmer *et al.* (1992)
Pasteurella piscicida	0.01 - 1.56	Palmer *et al.* (1992)
Pseudomonas fluorescence	3 - 6.25	Ledo *et al.* (1987); Palmer *et al.* (1992)
Pseudomonas putida	1.56	Palmer *et al.* (1992)
Vibrio carchariaiae	0.3	Ledo *et al.* (1987)
Vibrio damsela	0.075	Ledo *et al.* (1987)
Vibrio ordalii	3	Ledo *et al.* (1987)
Vibrio salmonicida	0.01 - 0.48	Martinsen *et al.* (1992)
Vibrio tubiashii	1	Ledo *et al.* (1987)
Yersinia ruckeri	0.075 - 0.312	Ledo *et al.* (1987); Ceschia *et al.* (1987)
Gram-positive bacteria		
*Enterococcus seriolicida***	50 - >100	Palmer *et al.* (1992)
Staphylococcus epidermis	1.56 - 6.25	Palmer *et al.* (1992)
Streptococcus iniae	25 - 50	Palmer *et al.* (1992)

* Formerly named *Vibrio anguillarum*; ** Formerly named *Streptococcus seriolicida*.

Flumequine was introduced into human medicine in the seventies. It has been used on a small scale, mainly for treatment of urinary tract and enteric infections (Rohlfing *et al.*, 1976; Rohlfing *et al.*, 1977). Today, this drug has only minor significance for human medicine due to the limited therapeutic utility and the availability of much more potent newer quinolones (Siporin, 1989; Vancutsem *et al.*, 1990).

Since the early eighties flumequine has been applied for fish disease control (Michel *et al.*, 1980; Chevalier *et al.*, 1981; Rodgers and Austin, 1983; Scallan and Smith, 1985; Takahashi *et al.*, 1990; Alderman and Michel, 1992) and its use is still increasing (cf. Figure 4). Flumequine is often used as a substitute for the non-fluorinated quinolone oxolinic acid, which penetrated the aquaculture market about 10 years earlier (Endo *et al.*, 1973a; Endo *et al.*, 1973b). At first, this shift will have been based on observed differences in clinical responses. However, an increasing number of laboratory studies demonstrated a higher *in vitro* antibacterial activity of flumequine for bacterial fish pathogens (Ledo *et al.*, 1987; Nakano *et al.*, 1989; Barnes *et al.*, 1991; Barnes *et al.*, 1992; Martinsen *et al.*, 1992; Palmer *et al.*, 1992). Moreover, comparative studies in Atlantic salmon showed that flumequine possesses more appropriate pharmacokinetic properties in this species (Martinsen *et al.*, 1993; Rogstad *et al.*, 1993).

Oxytetracycline

Oxytetracycline (Figure 5) is produced by strains of *Streptomyces* spp. and was first isolated by Finlay *et al.* (1950). This agent belongs to the group of tetracyclines and was the first true broad spectrum antibiotic, effective against a wide range of micro-organisms including Gram-positive and Gram-negative bacteria, *Rickettsiae*, *Mycoplasma* and *Chlamydia*. Oxytetracycline is a bacteriostatic drug, inhibiting prokaryotic protein synthesis by binding to the 30 S sub-unit of the bacterial ribosomes and thus preventing addition of amino acids to the growing peptide chain (Sande and Mandell, 1985).

Shortly after the introduction into human medicine, it appeared that oxytetracycline could also be effectively used in fish culture (Alderman, 1988). Thus this drug rapidly became the drug of choice in treatment of bacterial fish diseases. The frequency of oxytetracycline resistant bacterial strains has increased dramatically since its first application. Presently, the mainly R-plasmid associated tetracycline resistance is the most common resistance determinant among all bacterial species. In addition, exposure to tetracyclines may lead to co-resistance against other, chemically unrelated,

antimicrobial drugs (Levy, 1984). These unfavourable properties, together with the availability of more potent antibacterial drugs, will have played a crucial role in the declining utilization of this drug (Samuelsen, 1993).

Pharmacokinetics

Baggot (1977) defined pharmacokinetics as the mathematical description of concentration changes of drugs (and/or their metabolites) within the body following administration. A common approach for studying the pharmacokinetic behaviour of drugs is to depict the body as a system of distribution compartments. The latter are theoretical entities, usually without a clear physiological meaning. The number of compartments needed to adequately describe the disposition of a drug is dependent upon the course of the drug concentration in a particular situation. The body may be regarded as a single compartment if a drug distributes and equilibrates immediately between the different tissues. For other situations a 2 or more compartment model has to be used. In many instances the two-compartment open model (Figure 6) appears to be suitable (Baggot, 1977). It is assumed that a drug entering the body distributes instantaneously and homogeneously into the central compartment. For many drugs this consists of the blood plasma and extracellular fluid of readily accessible (highly perfused) organs. Distribution into the less well-perfused parts of the body, the peripheral compartment, is more slowly. Simultaneously, elimination (i.e. biotransformation and excretion) of the drug takes place from the central compartment.

In fish, like in other organisms, the fate of a drug is determined by its absorption, distribution and elimination. Besides being interdependent, these processes are affected by a variety of factors (Rasmussen, 1988; Ingebrigtsen, 1991; Nouws *et al.*, 1992), which may largely be classified as physico-chemical characteristics of the drug (e.g. ionization, molecular size and solubility), intrinsic factors related to the host (e.g. biotransformation and excretion capacity) and environmental circumstances (e.g. water temperature and salinity). The importance of the latter has to be emphasized, because, unlike in homeothermic animals, the physiological conditions for drug absorption and disposition in fish vary considerably depending on the environment (Smit *et al.*, 1981; Ishida, 1992). In particular the influence of temperature on pharmacokinetic parameters deserves special attention (Björklund *et al.*, 1992; Sohlberg *et al.*, 1994). In fish and

other poikilothermic animals, body temperature and associated metabolic rate hinge on ambient temperature (Ellis *et al.*, 1978). Therefore, biotransformation and active (excretion) processes are usually enhanced at higher temperatures. Furthermore, temperature interferes with translocation of drugs through an effect on membrane composition and blood flow.

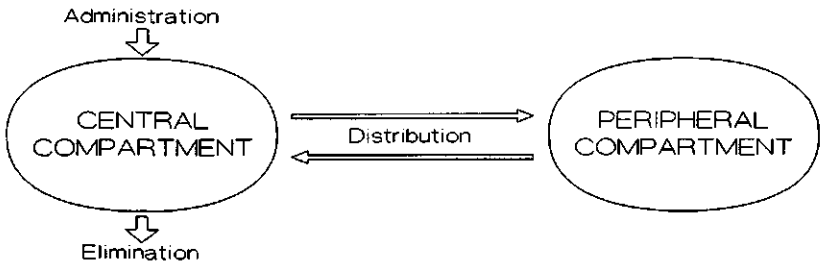

Figure 6. Concept of the open two-compartment model. After administration, the drug enters the system via the central compartment, where it distributes instantaneously. Distribution between central and peripheral compartments takes place more slowly. Elimination, which comprises biotransformation and excretion, is assumed to occur exclusively from the central compartment (adapted from Baggot, 1977; Ritschel, 1992).

Differences between fish species with regard to anatomy and physiology are often underestimated. Despite obvious similarities among these animals, mainly enforced by the characteristics of the aquatic environment, the wide variety of habitats that have been occupied, and the associated specific adaptations, account for a large diversity within this group. Resulting species-dependent differences in pharmacokinetics implicate that extrapolation of data from one fish species to another may be invalid (Ingebrigtsen, 1991; Nouws *et al.*, 1992). This may be illustrated by the plasma concentration versus time curves of intramuscularly injected oxytetracycline in three warm water species as shown in Figure 7. Except for the indicated temperatures, identical experimental conditions and analytical methods were employed. Most striking is the deviant elimination rate of the drug in European eel (*Anguilla anguilla* L.,1758; $t_{½}$=215 h) compared to common carp (*Cyprinus carpio* L., 1758; $t_{½}$=78.6 h) and African catfish (*Clarias gariepinus* Burchell, 1822; $t_{½}$=74.4 h), which can not be explained by the differences in ambient temperature alone.

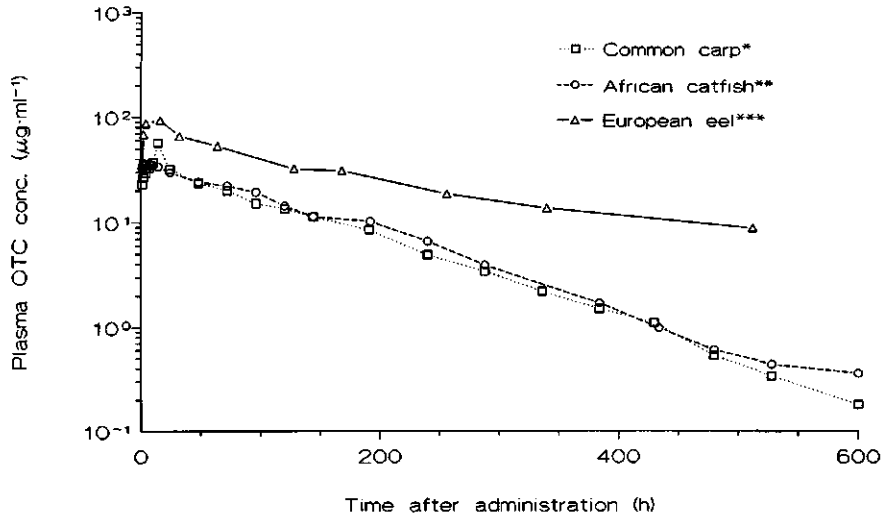

Figure 7. Mean concentrations of oxytetracycline (OTC) in plasma of common carp (*Cyprinus carpio* L., 1758) at 20 °C, African catfish (*Clarias gariepinus* Burchell, 1822) at 25 °C and European eel (*Anguilla anguilla* L., 1758) at 22 °C following a single intramuscular injection of 60 mg·kg^{-1} body weight. * Data from Grondel *et al.*, 1987a; ** data from Grondel *et al.*, 1989; *** data from Nouws *et al.*, 1993.

At present the available pharmacokinetic data of drugs in fish are limited. However, an increasing attention for this subject is observed in recent years, which is probably due to the rapidly growing need for safe and effective disease treatment methods in aquaculture (Schnick, 1992). The research is concentrated on the economically most important species (mainly salmonids), while species with a more regional, minor significance receive relatively little attention. Consequently, in general the scientific basis for the use of drugs in aquaculture is still insufficient. Therefore, treatment regimes and withdrawal periods are necessarily often based on those used in homeothermic animals (Boon *et al.*, 1991). This is an undesirable situation, because pharmacokinetic parameters may differ profoundly between fish and mammals or birds (Grondel *et al.*, 1986; Nouws *et al.*, 1986; Ingebrigtsen, 1991).

The immune system of teleost fish

Like other vertebrates, fish possess an extensive defence system, which enables the individual to survive and maintain its integrity in a hostile environment. The protective mechanisms are directed against foreign material recognized as non-self, including pathogens and malignant cells, and comprise a number of innate (non-specific) and adaptive (specific) reactions. The defence system of fish seems to be less complex than that of higher vertebrates (Ellis, 1982; Ratcliffe and Millar, 1988), but it will be very well adapted to the aquatic environment by natural selection during more than 350 million years. Although the immune system of fish is still less unravelled, the functional and morphological similarities with its mammalian counterpart are obvious. Correspondingly, fish contain most, if not all, of the different types of leucocytes found in mammals (Rowley *et al.*, 1988).

The non-specific immunity is characterized by the absence of an enhanced reaction after repeated infection (no memory formation) and the non-specific character. The non-specific defence in teleosts may be arranged according to the successive lines of defence encountered by invading substances (Lamers, 1985). In this way, the first line of defence comprehends the relatively stable physical and chemical barriers, which prevent penetration of foreign material into the host or eliminate these substances immediately after penetration. The epithelia, their secretions and several secreted and/or excreted protective (glyco)proteins (e.g. precipitins, agglutinins and lytic enzymes) are the most important factors in this line. The second line of non-specific defence consists of those processes that are more or less inducible upon infection. The cells involved are non-specific cytotoxic cells besides phagocytosing monocytes/macrophages and granulocytes. In addition, substances like complement factors and anti-viral and acute phase proteins play an important role at this stage.

Specific immune responses (accomplished by the immune system in its strict sense), which may be regarded as a third line of defence, include the reactions of lymphoid cells besides the cellular and humoral components that are related to the response of these cells. This form of defence is typified by antigen specificity and memory formation, which finds expression in an enhanced response after repeated contact with the same antigen. In teleost fish, the mechanisms involved in specific immunity seem to be essentially the same as in mammals and can accordingly be subdivided into cellular and humoral responses (Rijkers, 1981; Secombes, 1991). Likewise, strong

indications are available by now for the existence of mucosal immune responses in teleosts (Rombout *et al.*, 1993a; Rombout *et al.*, 1993b).

Humoral immune responses involve antigen uptake from the circulation or mucosae, followed by processing and presentation of the antigen by macrophages to antigen sensitive lymphocytes. Under influence of rather complex interactions between distinct leucocyte (sub)populations and immunoregulatory factors (cytokines; Secombes, 1991) produced by the interacting cells, B-lymphocytes proliferate and differentiate into antibody (immunoglobulin; Ig) secreting plasma cells. The specific antibody binds to the antigen, which can subsequently be eliminated by way of several mechanisms (Roitt *et al.*, 1993). Teleost fish produce only one class of Ig (physico-chemically resembling mammalian IgM), which however functionally mimics several mammalian Ig classes (Ellis, 1982).

Cell mediated immunity is attributed to the activity of a T-lymphocyte subpopulation termed cytotoxic T cells and it implies lysis of cells, recognized as non-self, by cell to cell contact. This mechanism plays a role in resistance against, for instance, virus-infected and neoplastic cells and in graft rejection. After activation, initiated by recognition of antigen expressed on cell membranes, T-lymphocytes can either act as cytotoxic T cells or as regulatory cells in humoral and cellular responses (T helper cells) by producing appropriate cytokines. Regulation and control of T-lymphocyte activation in teleosts is ascribed to a regulatory system analogous or homologous to the mammalian histocompatibility complex (Stet and Egberts, 1991). At present, different T-lymphocyte types are functionally characterized in several teleost species but they can not be well identified immunocytochemically because monoclonal antibodies, reactive to specific T cell membrane markers, are still not available (Koumans-van Diepen, 1993).

Immunomodulation

Immunomodulation is a consequence of a change in quantity and/or function of cells or substances involved in non-specific or specific immune responses. It thus concerns induction, blocking, amplification or inhibition of consecutive steps in defence reactions. Strictly speaking, the reactions against an antigen, which may imply both active immune responsiveness and tolerance, as well as the tuning of these responses by

immunoregulatory mechanisms, may be interpreted as immunomodulation. Moreover, the immune capacity of poikilothermic animals like fish, is highly dependent on ambient temperature. Within the temperature range of the species, higher temperatures usually enhance the response, while lower temperatures are suppressive (Rijkers *et al.*, 1980a). However, in the present thesis the term immunomodulation is used in connection with immunotoxicological events, which affect the defensive potential or the actual functioning of the defence system. This may result from any interference of foreign agents with the immunocompetent cells or the complexly organized communication between them. Disturbances may lead to immunosuppression, which negatively affects the health of the host by creating a deficient resistance to diseases. Conversely, stimulation of the defence system may also occur. This phenomenon obviously holds great potential for practical applications in disease prevention (Anderson, 1992; Onarheim, 1992).

Modulation of the defence system of fish may result from the action of environmental factors on critical physiological pathways. Depending on timing, duration and dose, exposure to ionizing radiation (Cosgrove *et al.*, 1975; Fabacher *et al.*, 1994) and environmental pollutants, such as pesticides and metal ions (Dunier, 1994) have frequently shown to impair fish immune systems. Until recently, relatively few experiments have been described in which immunostimulation was concerned. Most reports deal with adjuvants that have been added to fish bacterins to augment the intensity of the reaction, to increase the speed, and to prolong the duration of the specific immune response. However, there is an increasing interest in products that can be given alone (i.e. without simultaneous antigenic stimulation) and additionally induce a more generalized protection by boosting non-specific defence mechanisms. Special attention is paid to natural substances like glucans, which can be administered as feed additive and which may be easily certified by governmental agencies (Anderson and Siwicki, 1994).

Another category of possible immunomodulating substances is formed by the drugs used for prophylaxis and treatment of fish diseases. Antibacterial drugs are by far the most important drugs used in fish culture. Until about a couple of decades ago, surprisingly little was known about interactions of these drugs with defence mechanisms. Previously it has always been taken for granted that antibacterial drugs help the organism in its fight against infection by killing bacteria or suppressing their growth. By now, many reports have described either positive, negative or no effects at all of

antibacterial drugs on different aspects of the defence system (Grondel and van Muiswinkel, 1986). However, it is difficult to draw general conclusions because the picture is based on effects of different drugs studied in many animal species by means of a variety of tests. Moreover, the exact mechanisms of immunomodulation are often unknown or poorly understood (Van Loveren and Vos, 1991).

Only a limited number of studies is available concerning the effects of antibacterial drugs upon protective mechanisms and the associated health in fish. Immunomodulating characteristics of oxytetracycline, probably the most extensively studied drug in this respect, were investigated in common carp (*Cyprinus carpio* L., 1758). Injections with this drug significantly prolonged the survival of allografts (Rijkers *et al.*, 1980b). Grondel and Boesten (1982) found that the *in vitro* mitogenic response of leucocytes from several lymphoid organs to T cell mitogens showed an oxytetracycline-dependent decrease confirming the effect of this drug on T cell functions. Moreover, a suppressive effect of oxytetracycline on the *in vivo* humoral response in carp was described by Rijkers *et al.* (1980b; 1981) at the level of antigen binding and antibody producing cells. Numbers of antibody producing cells were reduced up to 95% during a primary response, while secondary responses were not significantly inhibited. Later it was demonstrated that the effect on the humoral response was more a delay than a suppression (Grondel *et al.*, 1987b). Negative effects of oxytetracycline on antibody production were also demonstrated in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792), whereas a potentiated sulphonamide (Ro5-0037), tested by the same regimes, did not affect this mechanism (Anderson *et al.*, 1984).

Grondel *et al.* (1985) tested various concentrations of several other antibacterial drugs for their possible interference with the mitogenic response of isolated pronephric leucocytes from carp in an *in vitro* system (Figure 8). From the results it can be concluded that the effect of most drugs is concentration-dependent. For different drugs either stimulation or suppression of the mitogenic response was observed. Moreover, some drugs were stimulators at lower concentrations whereas at higher doses they became suppressive. Taking into account that the distribution of antibacterial drugs across the body may be heterogeneous, depending on for instance the composition and blood perfusion of the tissues/organs and the biochemical characteristics of the drug (Ritschel, 1992), critical drug concentrations may be reached at immunologically important sites. Accumulation of a drug in particular tissues/organs may increase the probability for the emergence of lesions in the organs involved. For example Grondel

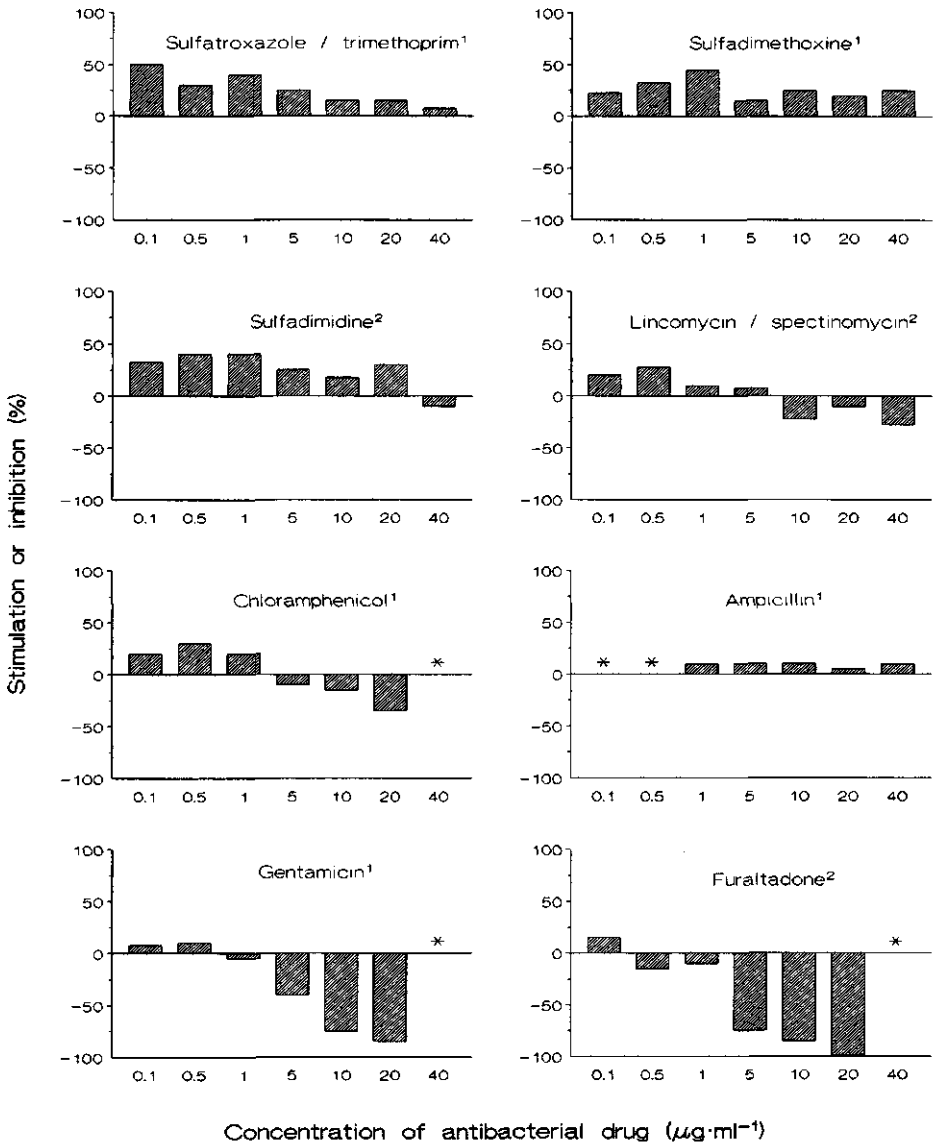

Figure 8. Several antibacterial drugs were tested for their possible interference with DNA synthesis. Phytohaemagglutinin-activated pronephric leucocytes from common carp (*Cyprinus carpio* L., 1758) were cultured for 4 days in the absence or presence of varying concentrations of different antibacterial drugs. The results of triplicate cultures are expressed in percentage stimulation or inhibition compared with mitogen-treated control cultures. [1]Injectable, [2]powder and * not tested (after Grondel *et al.*, 1985).

et al. (1987a) showed that oxytetracycline is accumulated and retained in scales, bone and lymphoid tissues in common carp. They suggested that the negative influence of oxytetracycline on carp immune responses may be related to the specific affinity of this drug for lymphoid tissues.

Immunomodulation can also be caused by an indirect effect of drug treatment on the defence system. For example drug interference with feed digestion or absorption may act upon the nutritional status and subsequently influence the animal's resistance (Blazer, 1992). Moreover, physical or chemical stress, which may result from drug treatment (e.g. handling of fish during the administration procedure or changes in water quality as a consequence of drug addition), is known to affect a variety of immunological parameters (Anderson, 1990). Stress initially affects the endocrine system by inducing higher levels of plasma cortisol (Pickering, 1992). Since the immune system is under hormonal control, including corticosteroids, these changes may in turn lead to alteration of defence mechanisms (Ellis , 1981).

As explained in the previous paragraph, immune responses are rather complex interactions between distinct leucocyte types and different cytokines, produced by the interacting cells. Therefore, major disturbances in immunological responses may result from a subtle modification at a crucial point. Still, resistance against an infection can be achieved by means of several routes and suppression or impairment of one route will not always lead to a recognizable pathology. Furthermore, different leucocyte populations may have a variable sensitivity to toxic agents. This complexity and multiplicity of immune responses often make a single test inadequate for evaluating immunomodulation and thus in most cases a battery of tests is recommended (Dunier *et al.*, 1994). Another approach, which also indicates the practical relevance of a possible immunomodulation, is the challenge with a pathogen. By using these 'real world' tests the defence system's function is more integrally addressed, as resistance against pathogens is generally based on a series of, often mutually dependent, non-specific and specific protective mechanisms (Van Loveren and Vos, 1991).

Indicator assays

To monitor immunomodulation in fish many *in vitro* and *in vivo* immunological assays are available, which are mainly a result of adaptation of techniques originally developed for mammals. These assays can be divided into three broad categories (Table

2). The general assays are *in vivo* tests indicating the health of fish by measuring parameters that report the potential and global condition of the defence system without giving information about an effect on a specific immune mechanism. Except for the challenge test, these assays are normally used without antigenic stimulation. The assays of non-specific resistance used in fish mainly comprise tests for the functional activity of the cells involved in the non-specific defence or the initial steps of a specific response. These tests are carried out *in vitro* and can be used with and without prior *in vitro* or *in vivo* stimulation. The last category of immunomodulation indicator assays consists of both *in vitro* and *in vivo* tests that usually include antigenic or mitogenic stimulation and subsequent measurement of specific immune response mechanisms.

Table 2. Selection of (immunological) indicator assays used to determine immunomodulation in fish

Assay and/or parameter	Specific modulation	Reference (example)
General assays		
Weight of lymphoid organs	None	Anderson *et al.* (1982)
Haematocrit (blood cell level)	None	Elsaesser and Clem (1986)
Leucocrit (white blood cell level)	None	Pickering and Pottinger (1987)
Leucocyte differentials	None	Blaxhall (1972)
Total/differential plasma protein level	None	Holmberg *et al.* (1972)
Challenge with pathogen (host survival; pathogen recovery)	Protection against disease	Houghton and Matthews (1986)
Non-specific resistance assays		
Phagocytic index	Endocytosis	Olivier *et al.* (1986)
Chemiluminescence (oxidative activity)	Phagocyte killing potential	Dunier *et al.* (1994)
Cytotoxic cell activity	Natural cytotoxicity	Rougier *et al.* (1994)
Specific immune response assays		
Blastogenisis (mitogenic response; mixed leucocyte response)	Lymphocyte proliferation	Grondel *et al.* (1985)
Allograft rejection	Cellular immunity	Rijkers *et al.* (1980)
Hemolytic plaque assay	Antibody production	Rijkers *et al.* (1981)
ELISPOT assay	Antibody production	Dunier and Siwicki (1994)
Agglutination	Antibody production	Grondel *et al.* (1987b)
ELISA	Antibody production	Rijkers *et al.* (1981)

Modified from Anderson, 1990.

Specific immune responses and in particular the production of circulatory antibodies, are among the most frequently used determinants of immunomodulation. Indeed, measurement of the presence and amount of humoral antibodies include some of the most sensitive and fastest immunoassays with the added advantage that the results are often linked to the degree of protection against a specific disease (Anderson, 1990; Van Loveren and Vos, 1991). However, one should be aware that modulation of a specific response, which is measured in the final phases of an immune reaction, may be due to an effect on earlier processes in the sequence of defence mechanisms.

The assessment of (functional) characteristics of the immune system generally benefits very much from the availability of monoclonal antibodies (mAbs) recognizing cell-specific membrane antigens or products. These mAbs are accurate and highly specific tools that can be used in several qualitative and quantitative tests, including microscopic and flow cytometric analyses of cells or enzyme-linked immunosorbent assays (ELISAs) for detection of certain (immunologically important) serum constituents.

In recent years there is a trend towards the use of *in vitro* models for studying immunomodulation, which is aimed at a reduction of experimental animals and (toxic) test chemicals. Although this may be a valuable approach to study the mechanisms of immunomodulation, a serious drawback is the difficulty to extrapolate the results to the real *in vivo* situation. Under *in vitro* circumstances the complex interactions between the different cell types and cell products, which are characteristic for immune responses, are virtually lacking. Moreover, the actual changes of the concentration of a xenobiotic substance in an animal as a result of absorption and disposition processes, as well as possible metabolic activation of those substances, can not be implicated. In the present thesis preference is given to *in vivo* assays so as to obtain more relevant and readily applicable results for the aquacultural practice.

Aim and outline of the thesis

Application of antibacterial drugs has played and will certainly continue to play an important role in the control of bacterial diseases in intensive aquaculture. However, as yet the scientific basis for the use of these drugs is often lacking or incomplete. Research in this area is concentrated on the major species in large scale intensive

culture, notably salmonids. Attention is mainly paid to the pharmacokinetics and *in vitro* and *in vivo* efficacy of a number of drugs. For other species than those mentioned above the situation is generally worse. As yet in many cases the application of drugs is still based on empiricism and/or on knowledge from work with other, often unrelated fish species or even homeothermic farm animals. Investigations into safety issues, related to the use of antibacterial drugs, are limited to some residue studies and, more recently, a growing interest in the impact of drugs on the (still mainly marine) environment is observed. Only few studies deal with the effect of antibacterial drugs on fish health, although these drugs are known as potential immunomodulators.

The current thesis intends to contribute to the comprehensive task of establishing rational criteria for the use of antibacterial drugs in fish, which ultimately may promote sound and effective medical management in aquaculture. The studies presented are focused on European eel, because of the economical importance of this species in a number of countries, including The Netherlands. With respect to the antibacterial drugs, emphasis is clearly put on flumequine. This drug is one of the most recently introduced drugs in eel culture, which is commonly used and expected to gain importance in the future. Within this framework, the attention is concentrated on possible drug induced immunomodulating effects and the associated influence on fish health.

Positive as well as negative effects of drugs are partly dependent on the concentrations of the product and/or its possible metabolites in the host after administration. Accordingly, the first experiment described in this thesis deals with the kinetics of flumequine in 3 warm water fish species (**chapter 2**). Using a comparative approach, the plasma disposition of the drug was investigated in common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) following a single peroral administration. For the latter species the pharmacokinetic research is continued with the work presented in **chapter 3**. This chapter provides data on the distribution and residue elimination of flumequine in eel, which were obtained by determination of tissue levels of the drug after an intramuscular injection.

Chapter 4 relates to the production and subsequent characterization of monoclonal antibodies (mAbs) to immunoglobulin of the European eel. Within the present research these mAbs were essential to accurately investigate the effect of drug-treatment on several immunological parameters. In the following experiments they are used to

identify and enumerate immunoglobulin bearing lymphocytes (B cells) and to determine the antibody response after infection.

The interference of flumequine with the proliferative capacity of European eel lymphocytes is described in **chapter 5**. An *in vivo* lymphocyte stimulation (blastogenesis) assay was developed and attention was paid to the drug effect on both concanavalin A (Con A) and lipopolysaccharide (LPS) induced proliferation. Subsequently, in **chapter 6** an available challenge model was used to study the influence of flumequine and also oxytetracycline exposure on the overall condition of the eels defence system. The fish were experimentally infected with the parasitic swimbladder nematode *Anguillicola crassus* and the resistance against this infection was assessed on the basis of parasite recovery, numbers of different circulating leucocytes and specific antibody production.

Finally, in **chapter 7** the results of the preceding chapters are summarized and discussed in a broader context. Moreover, some recommendations for further research are indicated.

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Chapter 2

Plasma disposition of flumequine in common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) after a single peroral administration

M.H.T. van der Heijden[1], H.J. Keukens[2], W.H.F.X. van den Nieuwboer[1], M.J.B. Mengelers[2] and J.H.Boon[1]

[1] *Department of Fish Culture and Fisheries, Wageningen Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands*

[2] *State Institute for Quality Control of Agricultural Products, P.O. Box 230, 6700 AE Wageningen, The Netherlands*

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Abstract

The disposition of perorally administered flumequine in plasma was compared between common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) respectively, at a dose of 18 $mg \cdot kg^{-1}$ body weight. All species showed rapid absorption followed by biphasic plasma depletion. Significant interspecies differences were observed for most of the determined pharmacokinetic parameters. Mean highest plasma flumequine concentrations were 6136 $ng \cdot ml^{-1}$ in carp, 4074 $ng \cdot ml^{-1}$ in eel and 1179 $ng \cdot ml^{-1}$ in catfish. Mean distribution and elimination half-lives were 3.4 and 104.3 h for carp, 7.3 and 59.5 h for catfish and 56.7 and 451.2 h for eel. To achieve equivalent drug efficacy and to minimize the probability of residues as a result of medication, dosage regimes and withdrawal periods should be adapted for each species.

Key-words: Pharmacokinetics, Flumequine, Common carp, African catfish, European eel.

Introduction

High stocking densities in intensive aquaculture result in an increased prevalence of infectious diseases. Administration of antibacterial agents is an effective means of controlling bacterial diseases in cultured fish (Gratzek, 1983). Nevertheless, there is a growing concern about the safety of fish medication to public health (Bernoth, 1991), the environment (Kupka Hansen *et al.*, 1992) and target animals (Van der Heijden *et al.*, 1992).

In many countries, legislation regarding the availability and use of medicinal products in aquaculture is inadequate, because they were formulated with 'conventional' veterinary medicine in mind (Alderman and Michel, 1992). However, marked differences between drug behaviour in fish and other phyla like mammals and birds are clearly demonstrated in literature. Most prominent is the often extended elimination half-life of drugs in fish compared to homeothermic animals. This can be explained by the usually large differences in physiological, biochemical and morphological properties of those taxa (Nouws *et al.*, 1986). Furthermore, external factors such as ambient temperature, pH and salinity influence the pharmacokinetics of most drugs in fish (Rasmussen, 1988; Björklund *et al.*, 1992; Ishida, 1992).

Pharmacokinetic data of fish are difficult to compare as a result of the variety of experimental conditions. In addition, various drugs, routes of administration and dose rates make proper comparative studies difficult.

The present study compared the plasma disposition of flumequine, after a single peroral administration, in three important cultivated fish species in The Netherlands, namely common carp, African catfish and European eel. Flumequine is commonly applied for the control of bacterial diseases in these species.

Materials and methods

Fish

Common carp (*Cyprinus carpio* L., 1758) and African catfish (*Clarias gariepinus* Burchell, 1822) were raised at the Wageningen Agricultural University (Huisman, 1974; Hoogendoorn, 1983) without drug use while European eel (*Anguilla anguilla* L., 1758)

was obtained from a commercial eel farm, where the fish had not been treated with any antibacterial agent.

Groups of 5 clinical healthy, non-sexed fish of each species, with a mean weight of 451.2 ± 118 g (carp), 382.6 ± 66.1 g (catfish) and 195.5 ± 33.0 g (eel), were used. The fish were acclimatized on a 12 h dark/12 h light photoperiod in a 150-litre aquarium with aerated tap water of 24 ± 1°C, pH 8.0 ± 0.2 at a flow rate of 150 $l \cdot h^{-1}$ for 14 days prior to the experiment. The fish were not fed during the acclimatization nor during the experimental period to avoid differences in drug kinetics as a consequence of differences in nutritional status.

Individual fish were identified by a subcutaneously injected micro-transponder containing an unique code (Adam *et al.*, 1992).

Antibacterial treatment

An aqueous solution of flumequine (Flumiquil®, premix consisting of 50% (w/w) pure flumequine, 20% (w/w) lactose and 30% (w/w) sodium carbonate; Pitman-Moore, Haarlem, The Netherlands) was prepared at a concentration of 4.5 $mg \cdot ml^{-1}$. Fish were sedated with Hypnodil® (0.3 $ml \cdot l^{-1}$; Janssen Pharmaceutica B.V., Beerse, Belgium) for about 5 minutes and flumequine was administered perorally as a single dose of 18 $mg \cdot kg^{-1}$ body weight by intubation of the stomach (catfish and eel) or the first gut segment (carp).

Blood sampling

The fish were sedated and 0.5 ml blood samples were collected from the caudal vein, with a heparinized syringe at 0, 2, 4, 8, 12, 16, 24, 48, 96, 144, 192, 336, 480, 624, 768, 912 and 1056 h after administration of the drug. Blood samples were immediately placed on ice and subsequently centrifuged for 10 min at 1300×g and 5°C. Plasma was stored at -60°C pending analysis.

Sample analysis

The concentration of flumequine was determined in plasma, using the high performance liquid chromatography (HPLC) method as described in this section.

Furthermore, plasma samples with high flumequine levels (>1000 $ng \cdot ml^{-1}$) were analyzed for 7-hydroxy-flumequine and both glucuronidated flumequine and 7-hydroxy-flumequine (Mevius *et al.*, 1990).

Standards

Flumequine was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and 7-hydroxy-flumequine was obtained from Riker/3M (Zoeterwoude, The Netherlands). Stock solutions of 100 $\mu g \cdot ml^{-1}$ were prepared in 0.01 M sodium hydroxide. Working standard solutions were prepared by dilution with water.

Reagents

All chemicals were of analytical grade (Merck, Darmstadt, FRG). Subtilisin was purchased from Boehringer (Mannheim, FRG) and beta-glucuronidase/arylsulfatase from IBF (France). Subtilisin solution was prepared by dissolving 12.5 mg subtilisin in 100 ml Tris buffer 0.1 M (pH 10). The HPLC eluent consisted of 660 ml 0.02 M *o*-phosphoric acid, 200 ml acetonitrile and 140 ml tetrahydrofuran. A 0.001 M *o*-phosphoric acid solution, passed through a chromatographic column (10×2 cm) filled with amberlite XAD-2 (0.3-0.78 mm; BDH, Dorset, UK), was used for flushing the on-line concentration column.

Instrumentation and chromatographic conditions

The column-switching HPLC system consisted of a Gilson 231-401 automatic sampler (Gilson Medical Electronics, Villiers-le-Bel, France), two 6000A liquid chromatographic pumps (Millipore, Milford, MA, USA), a Rheodyne 7010 automatic sixport switching valve (Berkeley, CA, USA) and a Hitachi F 1050 fluorescence detector (Merck, Darmstadt, FRG). The analytical column was a 150×4.1 mm ID PRP-1 column (Hamilton company, Reno, NV, USA). The concentration column (10×2.1 mm ID) was packed with Serdolit AD-4 0.05-0.1 mm (Serva, Heidelberg, FRG).

The mobile phase flow was 1 $ml \cdot min^{-1}$ and the sample enrichment flow was 0.8 $ml \cdot min^{-1}$. The fluorescence detector was operated at an excitation and emission wavelength of 315 nm and 360 nm, respectively. The timed event sequence for sample enrichment and separation was as follows: 0 min, injection and start flushing the concentration column; 4 min, activate sixport valve for backflushing the compounds of interest to the analytical column; 11 min, reset sixport valve and equilibrate the

concentration column with flushing solvent; 20 min, next injection.

Sample preparation

Frozen plasma samples were thawed and centrifuged for 5 min at 7000×*g*. Plasma (75 µl) and subtilisin solution (75 µl) were pipetted into a reaction vial and incubated for 20 min at 55°C. After cooling, 50 µl of the extract was analyzed by HPLC.

Hydrolysis of conjugates

A volume of 50 µl plasma was mixed with 150 µl buffer solution (pH 5). Following addition of 50 µl beta-glucuronidase the sample was incubated for two hours at 37°C.

After cooling, 250 µl Tris buffer (pH 10) and 250 µl subtilisin solution were added. The mixed sample was incubated for 20 min at 55°C. After cooling, 100 µl of the extract was injected in the HPLC system.

Method characteristics

The mean recovery of flumequine after spiking blank plasma samples of the investigated species with 250 $ng \cdot ml^{-1}$, was 89.9% (CV=8.3%; n=12) and the correlation coefficient of the standard curve was 1.0000 over the range 1-10000 $ng \cdot ml^{-1}$. Recovery of 7-hydroxy-flumequine was in the same order of magnitude as found for flumequine (80-100%).

None of 40 different drugs checked for interferences could be observed in the chromatograms. The limit of quantification was set at 2 $ng \cdot ml^{-1}$, corresponding to approximately twice the noise level for blank samples.

Pharmacokinetic analysis

Plasma flumequine concentrations of each fish were analyzed by curve stripping (Baggot, 1977) using PKCALC, a computer program for pharmacokinetic analysis (Shumaker, 1986).

The highest flumequine concentration observed and the corresponding time after administration were read from the collected concentration versus time data of the individual animals. Total areas under the plasma drug concentration versus time curves (AUC) were determined by the linear trapezoidal rule in the PKCALC-program.

Statistical analysis

Mean and standard error of the pharmacokinetic parameters were calculated. Wilk-Shapiro/Rankit plots were used to examine whether the pharmacokinetic values of the individual animals per species conformed to a normal distribution. A Wilk-Shapiro statistic ≥0.900 was assumed for a normal distribution. Significance of differences in pharmacokinetic parameters between the species was tested with the two sample T-test in case of normality and otherwise by the non-parametric Wilcoxon rank sum test. For both tests a 90% confidence interval was used.

All statistical analyses were performed with the computer program STATISTIX (1985).

Results

Typical chromatograms of standard solutions, blank and spiked plasma are shown in Figure 1. The highest plasma flumequine concentrations observed were 6136, 1179 and 4074 $ng \cdot ml^{-1}$ for carp, catfish and eel respectively and were significantly different. These concentrations were consistently observed at 2 h after administration to carp and catfish. In eel however, the time to reach the highest drug concentration was between 2 and 16 h after administration and was, due to this large variation, not significantly different from both other species.

The declining part of the plasma flumequine versus time curve of the investigated species was characterized by a biphasic disposition profile which could be adequately described by the equation:

$$C_p(t) = Ae^{-\alpha t} + Be^{-\beta t}$$

where α is the distribution (first, faster disposition) slope, β is the elimination (second, slower disposition) slope, A is the intercept of the mono-exponential distribution slope α with the ordinate, B is the intercept of the back-extrapolated mono-exponential elimination slope β with the ordinate, and t is time. The observed individual plasma drug concentrations and the fitted disposition profiles of flumequine in carp, catfish and eel are depicted in Figure 2.

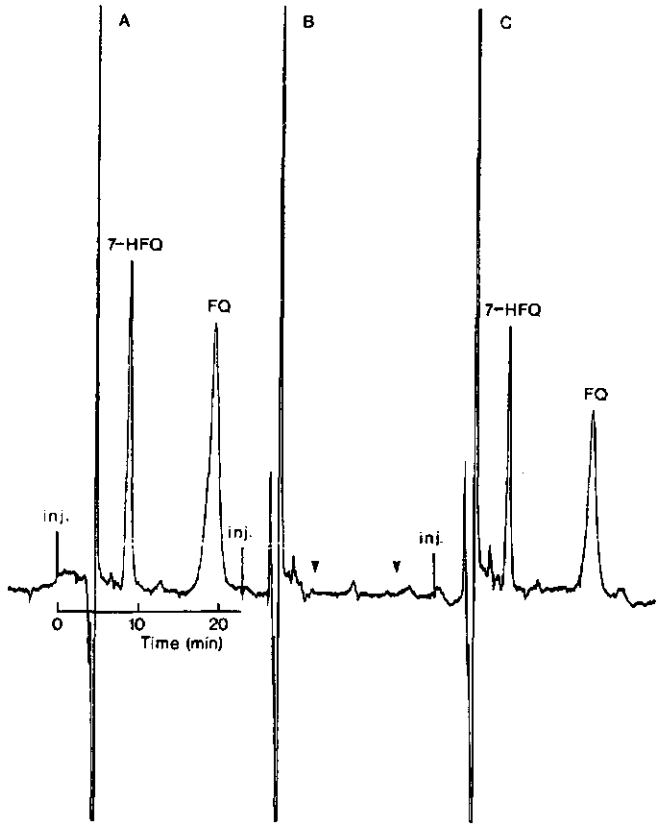

Figure 1. HPLC chromatograms of (A) standard solutions of flumequine (125 ng·ml^{-1}) and 7-hydroxy-flumequine (50 ng·ml^{-1}), (B) blank carp plasma extract and (C) extract of blank carp plasma spiked with 100 ng·ml^{-1} 7-hydroxy-flumequine and 250 ng·ml^{-1} flumequine. Peaks: 7-HFQ, 7-hydroxy-flumequine; FQ, flumequine.

The respective mean distribution ($t_{½\alpha}$) and elimination ($t_{½\beta}$) half-lives revealed 3.4 h (interval used for fitting: 2-24 h) and 104.3 h (interval: 48-480 h) for carp; 7.3 h (interval: 8-24 h) and 59.5 h (interval: 48-192 h) for catfish; and 56.7 h (interval: 4-144 h) and 451.2 h (interval: 192-1056 h) for eel. Mean values of selected pharmacokinetic parameters per species and the corresponding coefficients of variation are presented in Table 1.

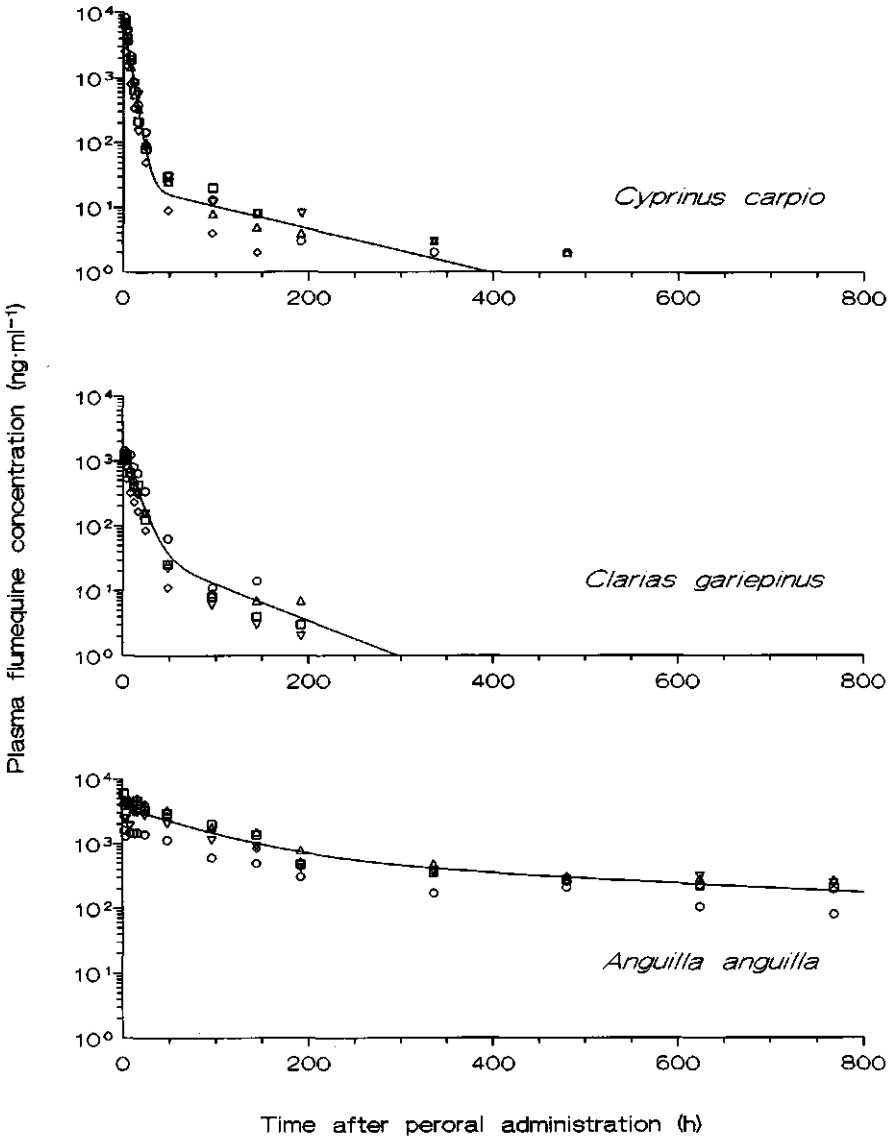

Figure 2. Plasma flumequine concentrations in the individual animals and mean disposition profiles in common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) following a single peroral administration of 18 mg·kg^{-1} body weight. During the acclimation and experimental period fish were kept at 24 ± 1 °C and were deprived of feed. Different symbols represent data of different fish. The curves represent fits obtained by curve stripping using the intervals 2-24 and 48-480 h for carp; 8-24 and 48-192 h for catfish; and 4-144 and 192-1056 h for eel.

Table 1. Pharmacokinetic parameters of flumequine in common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) (n=5 for each species) after a single peroral dose of 18 $mg \cdot kg^{-1}$ body weight

Parameter		Mean value and coefficient of variation (%)		
		Carp	Catfish	Eel
A	$(ng \cdot ml^{-1})$	8221 (35.5)	1447 (43.3)	3095 (41.6)
B	$(ng \cdot ml^{-1})$	23 (31.3)	44 (69.1)	639 (36.7)
α	(h^{-1})	0.2026 (7.2)	0.0961 (12.0)	0.0126 (21.1)
β	(h^{-1})	0.0078 (56.2)	0.0127 (31.0)	0.0016 (22.0)
$t_{½\alpha}$	(h)	3.4 (6.7)	7.3 (11.5)	56.7 (18.3)
$t_{½\beta}$	(h)	104.3 (36.6)	59.5 (34.7)	451.2 (29.5)
AUC	$(ng \cdot h \cdot ml^{-1})$	38307 (34.7)	16746 (40.5)	545250 (31.1)

Fish were kept at 24 ± 1°C and were not fed.

Table 2. Statistical comparison of pharmacokinetic parameters of flumequine in common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758) (n=5 for each species) after a single peroral dose of 18 $mg \cdot kg^{-1}$ body weight

Parameter		Two-tailed P-value		
		Carp vs catfish	Carp vs eel	Catfish vs eel
A	$(ng \cdot ml^{-1})$	0.008	0.056	0.033
B	$(ng \cdot ml^{-1})$	NS	0.008	0.008
α	(h^{-1})	0.008	0.008	0.008
β	(h^{-1})	0.056	0.008	0.008
$t_{½\alpha}$	(h)	0.008	0.008	0.008
$t_{½\beta}$	(h)	0.056	0.008	0.008
AUC	$(ng \cdot h \cdot ml^{-1})$	0.032	0.008	0.002

Fish were kept at 24 ± 1°C and were not fed. Significance of differences was tested with the two sample T-test in case of normality and otherwise by the non-parametric Wilcoxon rank sum test. NS, not significant (P>0.100).

The statistical comparison of the pharmacokinetic parameters is summarized in Table 2. Significant interspecies differences were observed for most of the determined parameters. However, the most pronounced differences were generally found between

eel and the other two species.

Only traces of 7-hydroxy-flumequine were found in any of the investigated species. The same held for glucuronide metabolites because no significant increase of the concentrations of flumequine and/or its 7-hydroxy metabolite were observed after deglucuronidation.

Discussion

The present study clearly demonstrates a species difference in the plasma disposition of flumequine after peroral administration. The 3 fish species were simultaneously exposed to identical external conditions, including an ambient temperature of 24°C. This temperature is within the range of the actually applied culture temperatures for the investigated species. Thus, it was possible to detect true species related differences and omit temperature influences with regard to drug disposition. However, at the temperature used, the relative metabolic rate will vary between the species. The observed pharmacokinetic differences might be less pronounced if the species were kept at their respective optimal temperatures for growth, indicating a more equal relative metabolic activity.

The fish were deprived of feed to avoid increased intra- and interspecies variation in drug kinetics as a consequence of differences in nutritional status. Previous observations revealed that the inevitable manipulation of the animals caused a loss of appetite and eels proved to be particularly susceptible. As a consequence, a large variation in feed intake was observed between and within species (unpublished results). Therefore, the procedure of not feeding the animals was unavoidable to ensure valid cross species comparisons.

The absorption of flumequine in the present study was rapid, so that not enough data were collected to characterize the absorption phase. The fast absorption might be explained by the administration of a dissolved drug without addition of any feed to fasting animals.

The most important differences between the investigated species were observed in the distribution and elimination of the antibacterial agent. Species-dependent physiological dissimilarities in excretory organs, tissue composition and vascularisation and protein binding could cause these differences in pharmacokinetics (Ingebrigtsen,

1991). Metabolites other than the 7-hydroxy and glucuronide metabolites may be formed and can not be excluded as a possible explanation for the difference in elimination rate.

Boon *et al.* (1991) found a slow biphasic disposition for flumequine following a single intramuscular administration of 9 $mg \cdot kg^{-1}$ body weight to eel. They attributed the long half-life of the first disposition phase partially to retarded absorption of the injected flumequine suspension. Absence of both, biotransformation and active excretion was suggested to explain the slow elimination.

Under the present experimental conditions therapeutic plasma concentrations against *Aeromonas hydrophila*, with a minimum inhibitory concentration (MIC) of 75 $ng \cdot ml^{-1}$ (Ledo *et al.*, 1987), were maintained for about 1 day in both carp and catfish and more than 44 days in eel. The slow elimination of flumequine from eel indicates a prolonged withdrawal time for treated fish destined for human consumption (Van der Heijden *et al.*, 1993). Moreover, it seems reasonable that lengthy exposure to the drug, might promote development of resistant bacteria (Bernoth, 1991) and eventual adverse side effects on the target animals health (Van der Heijden *et al.*, 1992).

Van Ginniken *et al.* (1991) reported species-related differences between the pharmacokinetic profile of sulphadimidine in carp and rainbow trout acclimated at 10 and 20°C. However, differences in mean body weight between the species in the latter study may have influenced the pharmacokinetic disparities. A possible effect of weight on pharmacokinetics within species, as demonstrated for some homeothermic animals (Lashev *et al.*, 1992; Nouws *et al.*, 1983), can also not be excluded with the experimental design of the present study.

Assuming a positive correlation between the plasma concentration of a certain drug and its efficacy, an equivalent therapeutic effect of flumequine in carp, catfish and eel can only be achieved if the dose size and dosing interval are adapted accordingly. The same applies to the establishment of withdrawal periods. Prevention of unwanted drug residues in fish is only possible if withdrawal periods are formulated per species. Taking the influence of species, temperature and nature of the drug on pharmacokinetics in fresh water fish species into account, three-dimensional matrices for dose regimes and withdrawal times are needed to conceptualize the therapeutically correct use of drugs in the species concerned.

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Chapter 3

Residue depletion of flumequine in European eel

M.H.T. van der Heijden[1], J.H.Boon[1], J.F.M. Nouws[2] and M.J.B. Mengelers[2]

[1] *Department of Fish Culture and Fisheries, Wageningen Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands*

[2] *State Institute for Quality Control of Agricultural Products, P.O. Box 230, 6700 AE Wageningen, The Netherlands*

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Abstract

Tissue distribution and residue elimination of flumequine, a quinolone derivative, was studied in European eel (*Anguilla anguilla* L., 1758) after a single intramuscular injection. Concentrations of flumequine in muscle and liver were respectively similar and higher than in plasma during the investigated period (44 days). Elimination of flumequine from the examined tissues was very slow and in the same order of magnitude as from plasma. After an observation period of more than 6 weeks the mean drug concentration in tissues and plasma was still above 85 $ng \cdot g^{-1}$ or $\cdot ml^{-1}$.

Key-words: Flumequine, Tissue residues, European eel.

Introduction

At present, a growing importance of eel culture within the Dutch aquaculture industry can be observed. The majority of these fish is kept in recirculation systems with high stocking densities at temperatures between 20 and 25°C (Heinsbroek, 1988). These conditions are associated with a high prevalence of bacterial infections and ask for an active disease control. Accordingly, antibiotics such as oxytetracycline and more recently flumequine, are administered on a large scale.

However, in The Netherlands, compounds specifically licensed for use in aquaculture together with appropriate dosage regimes, withdrawal periods and monitoring programs for any residues are not available as yet. Nevertheless, according to EC requirements the prescribing veterinarian has to ensure that a withdrawal period of at least 500 degree·days (withdrawal period in days = 500 degree·days divided by temperature in °C) is observed, in case of extra-label use of drugs (Bernoth, 1992).

Investigation of the pharmacokinetics of flumequine in eel plasma revealed that the disposition after intramuscular administration can be described by an open two-compartment model with an elimination half-life of about 255 h (Boon *et al.*, 1991). This indicates that the minimum withdrawal period stated by the EC might be too short to avoid unacceptable residues of flumequine in eels destined for human consumption.

To provide more rational criteria for the use of flumequine in eel culture, the persistence of flumequine in eel plasma and several tissues was determined during a 44 days period following a single intramuscular injection.

Materials and Methods

Twenty European eels (*Anguilla anguilla* L., 1758), with a mean weight of 469.6 g (range 350.4-647.0), received an intramuscular injection with a commercially available flumequine suspension (Fluject®, Dopharma B.V., Raamsdonkveer, The Netherlands) at a dose of 9 $mg \cdot kg^{-1}$ body weight. The fish were randomly divided into four groups of 5 animals. Each group was kept in a 150 l aquarium with aerated tap water of ± 23°C. Fish were deprived of feed.

At 48, 96, 192 and 1056 hours after injection one group of eels was sacrificed and plasma, bone, fat, posterior kidney, liver, muscle (non-injected region), skin and spleen

were sampled. Plasma and tissue samples were deep frozen at -20°C pending analysis of the drug concentration.

Furthermore, the longest-lived group was used to determine the concentration vs time profile in plasma by taking 17 blood samples of 0.5 ml from each animal during the time course of the experiment. The results of this part of the study have already been published (Boon *et al.*, 1991).

After thawing, the tissues were blendered and diluted 1:5 (w/v) with phosphate buffer (pH 7.4). Subsequently, an extraction procedure with dichloromethane was performed, the extract was evaporated and the dry matter was dissolved in eluent (125 ml acetonitrile, 200 ml dimethylformamide and 675 ml distilled water containing 3 g phosphoric acid and 1 g tetramethylammonium chloride). Thereafter, the samples were analyzed by means of high performance liquid chromatography (HPLC) using fluorescence detection (Boon *et al.*, 1991). With the proposed procedure it was possible to detect flumequine as well as its 7-hydroxy metabolite. The mean recovery of the parent compound for the measured range was >80% in plasma. In tissues it varied between 69.2% in skin to 87.8% in muscle. The limit of detection in plasma and tissues was 2 $ng \cdot ml^{-1}$ and 10 $ng \cdot g^{-1}$ respectively. Measured flumequine concentrations were corrected for the recovery in the corresponding tissue.

Results

The concentrations of flumequine in tissues and plasma of European eel after a single intramuscular injection are shown in Figure 1. The time to reach maximum concentrations of flumequine (t_{max}) in tissues (48-192 h) was comparable to t_{max} in plasma (96 h). Remarkably, t_{max} in bone and skin was achieved in about the same time as in plasma. Mean maximum concentrations in tissues ranged from 725 $ng \cdot g^{-1}$ in bone to 12100 $ng \cdot g^{-1}$ in fat. At the end of the observation period (t=1056 h) the mean drug concentrations in the investigated tissues and plasma were still clearly above the detection limit of the applied HPLC method; in decreasing order: liver (769 $ng \cdot g^{-1}$) > fat (427 $ng \cdot g^{-1}$) > muscle (238 $ng \cdot g^{-1}$) > plasma (213 $ng \cdot ml^{-1}$) > spleen (197 $ng \cdot g^{-1}$ > skin (153 $ng \cdot g^{-1}$) > bone (89 $ng \cdot g^{-1}$) > kidney (85 $ng \cdot g^{-1}$).

Elimination of flumequine from tissues was in the same order of magnitude as from plasma. However, from bone and skin the elimination was slower.

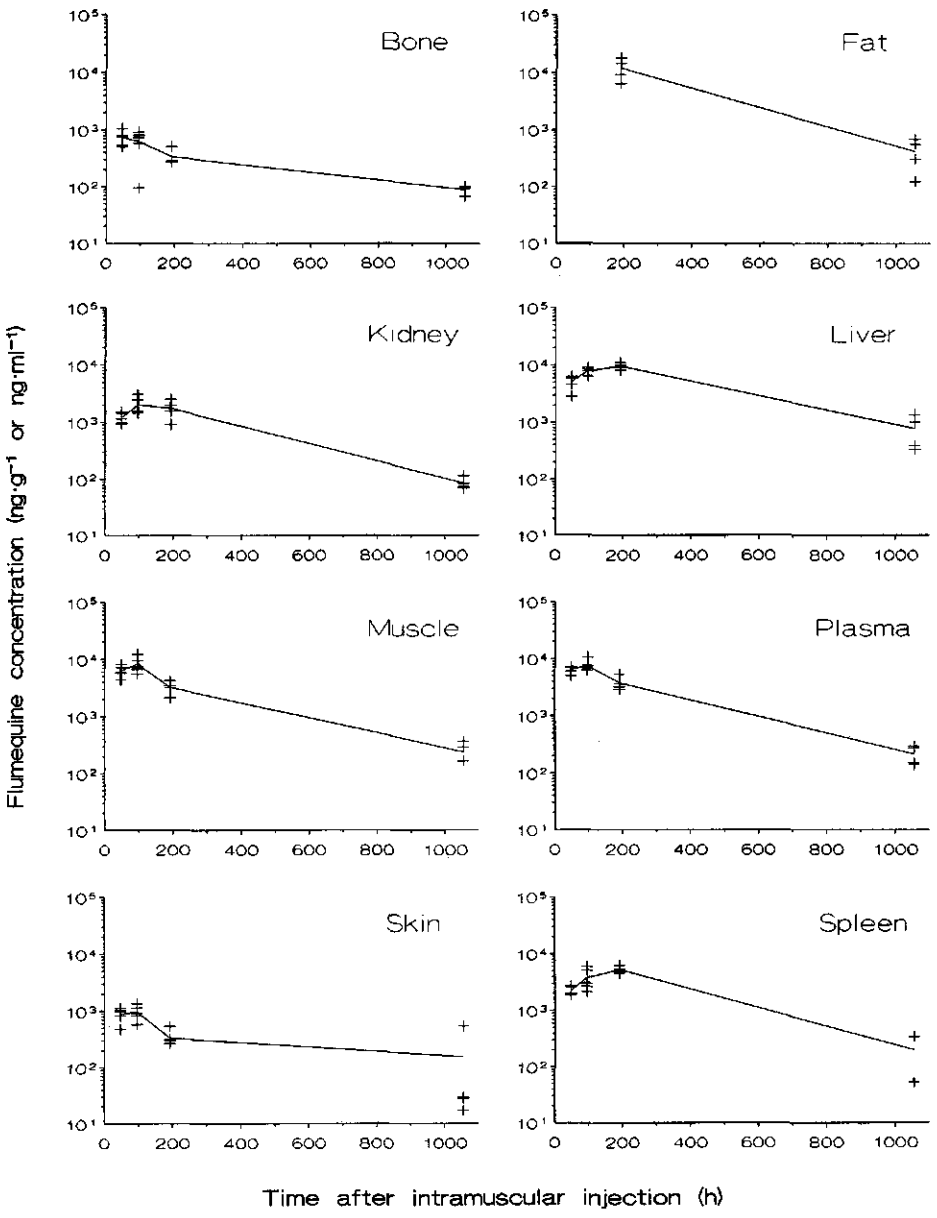

Figure 1. Scatter diagrams of flumequine concentrations in tissues of European eel (*Anguilla anguilla* L., 1758) following a single intramuscular injection of 9 mg·kg^{-1} body weight. Fish were kept at ± 23°C. The lines connect the means at the different sample times.

Ratios of tissue levels to the plasma level of flumequine are given in Table 1 in order to quantify the affinity of the drug for particular tissues of eel. Forty-eight hours p.i. the highest flumequine concentration was found in plasma. From that time onwards, flumequine concentrations in liver and fat, if determined, were higher than in plasma.

The mean muscle/plasma concentration ratio was 1.01 ± 0.10. Statistical analyses revealed a significant correlation ($r=0.993$; $P<0.05$) between both flumequine concentrations.

No 7-hydroxy-flumequine was observed in any of the analyzed samples.

Table 1. Ratios of mean tissue levels divided by the mean plasma level of flumequine in European eel (*Anguilla anguilla* L., 1758) after a single intramuscular injection at a dose of 9 $mg \cdot kg^{-1}$ body weight

Time	Tissue						
(h)	Bone	Fat	Kidney	Liver	Muscle	Skin	Spleen
48	0.11	ND	0.19	0.81	0.99	0.14	0.35
96	0.08	ND	0.26	1.04	1.08	0.13	0.50
192	0.09	3.20	0.46	2.54	0.85	0.09	1.39
1056	0.42	2.00	0.40	3.61	1.12	0.72	0.92

Fish were kept at ± 23°C. ND, not determined.

Discussion

The minimum inhibitory concentrations (MICs) of flumequine for most important eel pathogenic bacteria range from <0.08 to 0.3 $\mu g \cdot ml^{-1}$ (Ledo *et al.*, 1987). Concentrations of flumequine in plasma and in most of the tissues of European eel after intramuscular injection are higher than these MIC values for more than 44 days. This may explain the effective treatment of bacterial infections in eel after single dose administration of flumequine. Moreover, dose reduction without loss of effectiveness seems feasible. However, the long drug persistence in plasma and tissues may lead to undesirable residues of flumequine if a withdrawal period of 500 degree days (i.c. 22 days), which is in accordance with the EC directive, is applied. To evaluate the possible hazards for public health, maximum residue levels as well as properly designed dosage regimen studies and monitoring programs are needed.

Boon *et al.* (1991) mentioned the absence of biotransformation and active excretion as possible causes of the slow elimination from plasma. From the present results it is concluded that good tissue distribution and slow release from tissues, especially bone and skin, may also contribute to the slow elimination from plasma.

Because of the significant correlation between the drug concentration in muscle and plasma, the plasma flumequine concentration may provide a useful, simple tool for estimating residues in muscle of eel, which is the most important tissue with respect to human consumption.

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Chapter 4

Production, characterization and applicability of monoclonal antibodies to European eel (*Anguilla anguilla* L., 1758) immunoglobulin

M.H.T. van der Heijden[1], J.B.M.A. Rooijakkers[2], G.H.R. Booms[1], J.H.W.M. Rombout[2] and J.H. Boon[1]

[1] *Department of Fish Culture and Fisheries, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

[2] *Department of Experimental Animal Morphology and Cell Biology, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

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Abstract

Monoclonal antibodies (mAbs) to European eel (*Anguilla anguilla* L., 1758) immunoglobulin (Ig) were produced, characterized and tested for applicability in a number of immuno(cyto)chemical assays. The selected mAbs, WEI 1 and WEI 2, were specifically reactive with Ig heavy and light chain, respectively. WEI 1 appeared to react with all or nearly all Ig molecules, B cells and plasma cells. WEI 2 was reactive with a subpopulation of those cells, indicating that European eel possesses at least two antigenically different light chain types. Both mAbs could be used for detection of antigen-specific antibodies in plasma by means of an enzyme-linked immunosorbent assay (ELISA).

Key-words: Monoclonal antibodies, European eel, Immunoglobulin, B cells, ELISA.

Introduction

The growth of the aquacultural sector and the involved trade in living fish, increasingly asks for assessment of the health status of these animals. For this purpose definition of fish health parameters is required. At present, different parameters have been described, including those associated with behaviour, physiology and performance (Schreck, 1990), but more readily determinable haematological (Blaxhall, 1972) and immunological parameters (Anderson, 1990) are needed.

Immunological parameters, such as total and specific plasma immunoglobulin (Ig) levels and the number of B and plasma cells in different tissues, may be used to evaluate immunocompetence and associated disease resistance. Monoclonal antibodies (mAbs) specifically reacting with Ig provide a useful tool for proper determination of these immunological parameters.

During the last decade, mAbs have been produced to Ig of a number of teleost species (Lobb and Clem, 1982; Deluca *et al.*, 1983; Secombes *et al.*, 1983; Thuvander *et al.*, 1990; Sánchez *et al.*, 1993; Navarro *et al.*, 1993). This has greatly contributed to an improved understanding of the architecture and functioning of the immune system of teleost fish in general and the corresponding species in particular.

Unfortunately, mAbs to European eel (*Anguilla anguilla* L., 1758) Ig were not yet available, despite the growing economical importance of this predominantly intensively cultured species (Heinsbroek, 1991). The present study describes the production, characterization and applicability of mAbs specific to Ig of European eel using a number of immuno(cyto)chemical assays.

Materials and methods

Fish

Non-sexed European eels (*Anguilla anguilla* L., 1758), weighing 80 to 180 g, were either obtained from a local commercial farm or raised, from the glass eel stage onwards, at the Wageningen Agricultural University. Fish were maintained in aquaria with recirculating, aerated and filtered water of 24 ± 1°C. The diet consisted of pelleted eel feed (Provimi B.V., Rotterdam, The Netherlands). Blood was sampled from the

caudal, subvertebral vessels with a heparinized syringe, after fish were sedated in water containing Hypnodil® (Janssen Pharmaceutica B.V., Beerse, Belgium; 0.3 $ml \cdot l^{-1}$).

Purification of plasma Ig

Plasma samples of 25 eels were pooled and 10% (v/v) 1 M $CaCl_2 \cdot 2H_2O$ and 2% dextran sulphate 500 were added. After stirring 30 min on ice and centrifuging for 40 min at 10,000×*g*, the supernatant was precipitated twice during 4 h with saturated $(NH_4)_2SO_4$. Subsequently, the precipitate was dissolved in 0.01 M Tris buffer (pH 8.0) and loaded on a gel filtration column (Sephacryl® S-500; Pharmacia, Uppsala, Sweden). For selection, the collected Ig-containing fractions were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). Fractions with adequate purity were pooled and stored at -20°C.

Preparation of mAbs

A Balb/c mouse was injected intraperitoneally (i.p.) with 60 μg eel Ig in Freund's complete adjuvant (1:1; DIFCO, Detroit, MI, USA), 9 weeks later followed by i.p. injection of 100 μg eel Ig without adjuvant. Three days later the spleen was extirpated and a single cell suspension was prepared. Fusion of splenocytes with the mouse myeloma cell line Sp2/0-Ag-14 and subsequent culture and selection of hybridomas was performed according to the method described by Schots *et al.* (1992). Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) for production of anti-eel Ig antibodies of the IgG class. Briefly, microtiter plates coated with eel Ig were incubated with hybridoma culture supernatant and stained with alkaline phosphatase conjugated goat anti-mouse IgG (GAM IgG-AP; 1:5000; Sigma Chemical Co., St. Louis, MO, USA). Positive hybridomas were subjected to further selection using indirect immunofluorescence reaction on fixed cytocentrifuge slides of pronephros and spleen cells and flow cytometric analysis of isolated peripheral blood leucocytes (PBL) labelled by indirect immunofluorescence. The selected hybridomas were recloned and tested again by means of the procedures described above. The IgG isotype of selected mAbs was estimated by means of a sandwich ELISA, using rat anti-mouse Ig typing sera coated microtiter plates (Schots *et al.*, 1992).

Immunochemistry

Western blotting

SDS-PAGE of eel Ig and whole plasma was carried out using 4% gels or 7.5-15% gradient gels for separating native and 2-mercaptoethanol denaturated proteins, respectively. For estimation of molecular weights, prestained standards (Bio-Rad, Richmond, CA, USA) were used. After electrophoretic transfer of proteins onto nitrocellulose, transfers were soaked for 1 h in 0.02 M Tris/0.05 M NaCl (pH 7.4; TBS) containing 1% (w/v) BSA to block non-specific binding sites, washed in TBS containing 0.05% (v/v) Tween 20 (TTBS) and cut into strips. The latter were successively incubated for 1 h with 1:10 diluted anti-eel Ig mAbs and GAM-AP (1:3000; Bio-Rad, Richmond, CA, USA). Next, the strips were put in 100 mM Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$ and subsequently stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution.

Affinity chromatography

Anti-eel Ig affinity column was prepared by loading a column, packed with protein G-Sepharose® beads (Pharmacia, Uppsala, Sweden), with an anti-eel Ig mAb. Unbound material was removed by successively washing with TBS (pH 7.2), TBS (pH 4.5) and TBS/2 M NaCl (pH 8.0). Thereafter, purified eel Ig was applied onto the column and subsequently eluted with TBS (pH 4.5). From the application of anti-eel Ig mAb onwards, the flow through was evaluated for protein content by UV-absorbence at 280 nm and collected as 300 µl fractions. Thereafter, 100 µl of these fractions was analyzed for the presence of eel Ig by means of a dot blot assay, as described by Navarro *et al.* (1993).

Immunocytochemistry

Cytocentrifuge slides

Eel was exsanguinated and pronephros and spleen were extirpated. Thereafter, cell suspensions were prepared in 65% RPMI-1640 (Gibco, Breda, The Netherlands) containing 10 $IU \cdot ml^{-1}$ heparin, 0.1% (w/v) NaN_3 and 1% (w/v) BSA (eRPMI; pH 7.4, 240 $mOsm \cdot kg^{-1}$), by squeezing the tissues through a nylon gauze filter. Preparation and subsequent indirect immunofluorescence labelling of cytocentrifuge slides was carried

out as previously described by Navarro *et al.* (1993). Additional staining was performed by successively incubating the slides with two different anti-eel Ig mAbs (1:100), alternated with either tetramethyl rhodamine isothiocyanate or fluorescein isothiocyanate conjugated rabbit anti-mouse Ig antibodies (RAM-TRITC or RAM-FITC; 1:50; Dakopatts, Glostrup, Denmark). Slides were examined using a fluorescence microscope with phase-contrast equipment (Nikon, Microphot FXA).

Flow cytometry

Leucocytes with densities between 1.020 and 1.070 $g \cdot cm^{-3}$ were isolated from peripheral blood, pronephros and spleen cell suspensions on a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) density gradient. Cells (0.5×10^6) were washed, resuspended in 250 µl of different dilutions of anti-eel Ig mAbs and incubated for 30 min at 0°C. Subsequently, cells were washed, and incubated for 30 min at 0°C with RAM-FITC (1:100; Dakopatts, Glostrup, Denmark). Cell suspensions were analyzed with a FACStar® flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 5 W argon laser, tuned at 488 nm.

Immuno-electron microscopy

Eel PBL with densities between 1.020 and 1.070 $g \cdot cm^{-3}$ were isolated as described above. After washing, 4×10^6 cells were resuspended in 1 ml 1:100 diluted anti-eel Ig mAb, incubated for 45 min at 0°C, washed again, and incubated for another 45 min at 0°C with 100 µl goat anti-mouse Ig coupled to 15 nm gold particles (GAM-G15; 1:5; E·Y Labs inc., San Mateo, CA, USA). Cell pellets were prepared for electron microscopy as described by Van Diepen *et al.* (1991).

ELISA for detection of specific antibodies

Seven eels were immunized intramuscularly (i.m.) with 10 µg $DNP_{494}KLH$ (Calbiochem, La Jolla, CA, USA) in 50 µl phosphate buffered saline (PBS; pH 7.2), mixed with an equal volume Freund's incomplete adjuvant (DIFCO, Detroit, MI, USA). Simultaneously, 3 control eels received an i.m. injection with 100 µl PBS (pH 7.2). After 21 days control fish and three immunized fish were bled, plasma was obtained and stored at -20°C until detection of specific antibodies. At day 36 and 48, respectively, the remaining 4 fish were boosted and sampled according to the procedures described for

the priming injection. Antibodies to DNP-KLH or DNP alone were detected by means of an ELISA, essentially the same as described by Arkoosh and Kaattari (1990). Major modifications were the use of DNP_{494}KLH-coated plates next to DNP_{44}BSA-coated plates. Furthermore, 5% (w/v) lean milk powder in TTBS was used for blocking non-specific binding sites.

Results

Production and selection of mAbs

After the fusion, more than 30% of the 200 harvested clones produced antibodies of the IgG class, reacting with purified eel Ig. Additional screening of these clones by means of indirect immunofluorescence reactions on cytocentrifuge slides and flow cytometry, resulted in 30 positive clones, of which 15 were still positive after recloning and subsequent testing. Western blots of purified eel Ig, run under reducing conditions, yielded only 3 promising mAbs, one of them immunoreactive with the Ig heavy (H) chain, and the other 2 immunoreactive with the Ig light (L) chain. Because the latter 2 mAbs appeared to recognize the same population of lymphocytes, only 2 clones (WEI 1 and WEI 2) were selected for further use.

Characterization of mabs

Immunochemistry

Figure 1 shows the immunoreaction of WEI 1 and WEI 2 in Western blots of non-reduced and reduced purified Ig and reduced whole plasma. Eel Ig appeared to occur in a tetrameric, dimeric and monomeric form, which were all reactive with both mabs. Apart from that, WEI 1 and WEI 2 showed a specific reaction with the Ig H (± 70 kDa) and L chain (± 25 kDa), respectively.

Isotyping revealed that WEI 1 belongs to the IgG_1 subclass whereas WEI 2 was found to be of the IgG_{2a} subclass.

Affinity chromatography of purified eel Ig, by means of Sepharose® immobilized WEI 1, and subsequent analysis of the flow through by a dot blot assay using WEI 2, revealed no demonstrable Ig in fractions collected before saturation of the column.

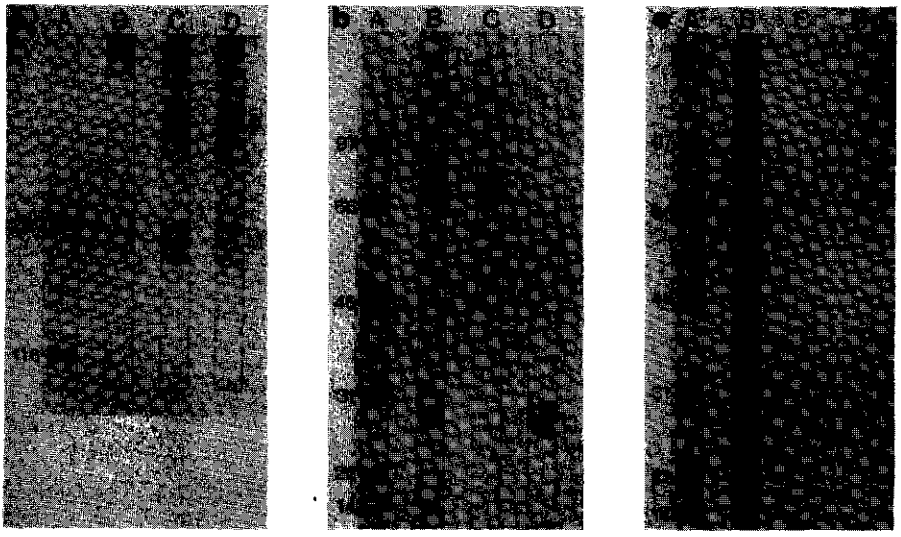

Figure 1. Western blots comparing the reactions of the anti-eel Ig mAbs. Lane A, amido black stained molecular weight markers with their respective molecular weights (kDa); Lane B, amido black stained sample; Lane C, immuno-stained with WEI 1; Lane D, immuno-stained with WEI 2. (a) Non-reduced Ig sample; (b) Reduced Ig sample; (c) Reduced whole plasma sample. T, tetramer; D, dimer; M, monomer; H, heavy chain; L, light chain.

Immunocytochemistry

Both anti-eel Ig mAbs showed specific immunoreaction in cytocentrifuge slides of pronephros and spleen cells. Surface labelling of a subset of the lymphocytes was observed, as well as strongly stained cytoplasm of plasma cells. Additional staining of pronephros cells with WEI 1 followed by WEI 2 and vice versa showed that WEI 2 reacted with a smaller number of cells than WEI 1 (Figure 2).

Table 1 summarizes mean percentages of Ig-bearing (sIg^+) cells in peripheral blood, pronephros and spleen cell suspensions, as measured by flow cytometry after labelling with WEI 1 or WEI 2. All 3 suspensions revealed around 25% sIg^+ cells with WEI 1, but much lower percentages (9.1-16.2%) with WEI 2. Figure 3 shows the forward/side (90°) scatter (FSC/SSC) dot plot of PBL and corresponding fluorescence histograms of a representative fish. Simultaneous incubation of PBL suspensions with equal volumes of WEI 1 and WEI 2 revealed no significant increase (one-tailed two sample T-test, $P=0.15$) of sIg^+ cells compared to suspensions labelled only with WEI 1.

Pre-embedding immunogold labelling of PBL with either WEI 1 or WEI 2 resulted in selective surface labelling of a subset of the lymphocytes. The gold particles were mainly found in clusters, although labelling intensity per cell was apparently higher after using WEI 2 compared to WEI 1 (Figures 4a and 4b).

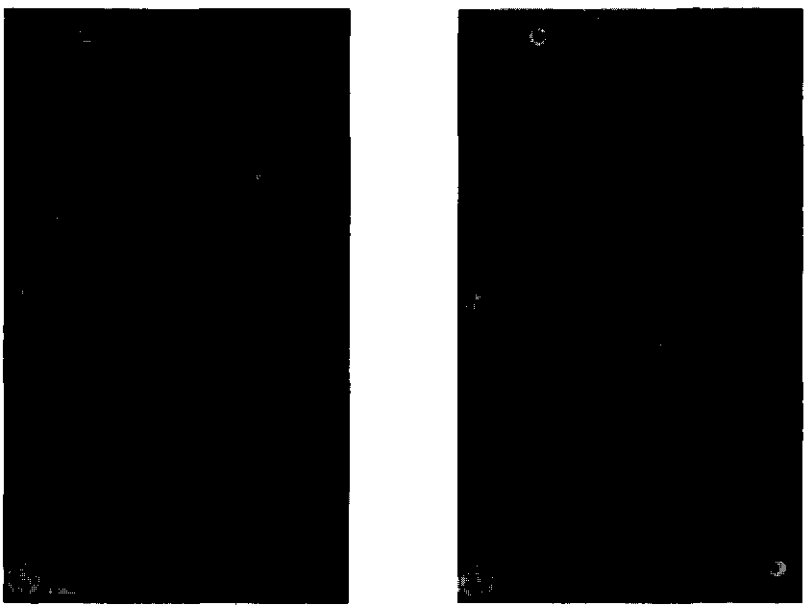

Figure 2. Fluorescence micrographs of a cytocentrifuge preparation of spleen cells immuno-stained with WEI 2 and RAM-FITC followed by WEI 1 and RAM-TRITC. Scale bar = 20 μm. (a) WEI 2-immunoreactive cells photographed with a FITC filter combination. (b) WEI 1 and WEI 2-immunoreactive cells in the same slide, photographed with a TRITC filter combination.

Table 1. Mean percentages (± SD) of Ig-bearing (sIg^+) cells labelled by indirect immunofluorescence with WEI 1 or WEI 2 in peripheral blood, pronephros and spleen cell suspensions

mAb	sIg^+ cells (%)*		
	blood (n=13)	pronephros (n=3)	spleen (n=3)
WEI 1	26.0 ± 6.2	24.0 ± 8.6	25.3 ± 11.7
WEI 2	16.2 ± 4.4	9.1 ± 2.7	13.1 ± 7.0

* Determined using gated, lymphoid cells as shown in Figure 3a.

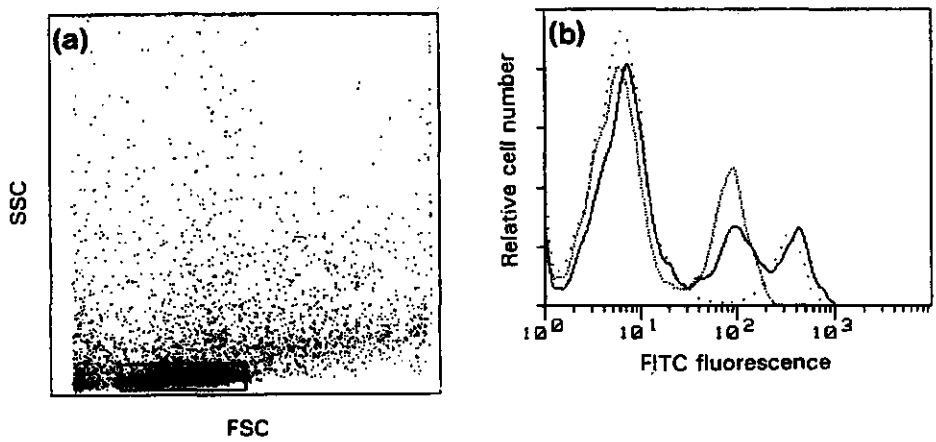

Figure 3. Flow cytometry generated forward/side (90°) scatter (FSC/SSC) profile and fluorescence histograms of PBL labelled by indirect immunofluorescence with WEI 1 and/or WEI 2. (a) FSC/SSC dot plot of PBL with gated, lymphoid cells; (b) Combined (smoothed) FITC fluorescence histogram of gated, lymphoid cells stained with WEI 1 (·····), WEI 2 (· · · ·) or with WEI 1 + WEI 2 (———).

ELISA for detection of specific antibodies

WEI 1 or WEI 2 gave similar results when applied in an ELISA for detection of DNP (Figures 5a and 5b) or DNP-KLH-specific (Figures 5c and 5d) antibodies in plasma of immunized eels. Especially in case of anti-DNP-KLH detection, low background values were recorded for plasma from sham-immunized fish. Animals immunized twice exhibited much higher mean titers than those immunized once.

Discussion

The mAbs produced and described in the present study were extensively selected to ensure an adequate, specific reaction in a broad range of immuno(cyto)chemical assays. Only mAbs of the IgG class were selected to avoid problems with clumping of cells during reactions on cell suspensions, as reported for antibodies of the IgM class by Van Diepen *et al.* (1991). The specificity of the selected mAbs is most clearly illustrated by the Western blots of whole plasma and the immunogold labelling of PBL. In both cases a very specific reaction was observed, while they were exposed to a great range of different antigenic determinants.

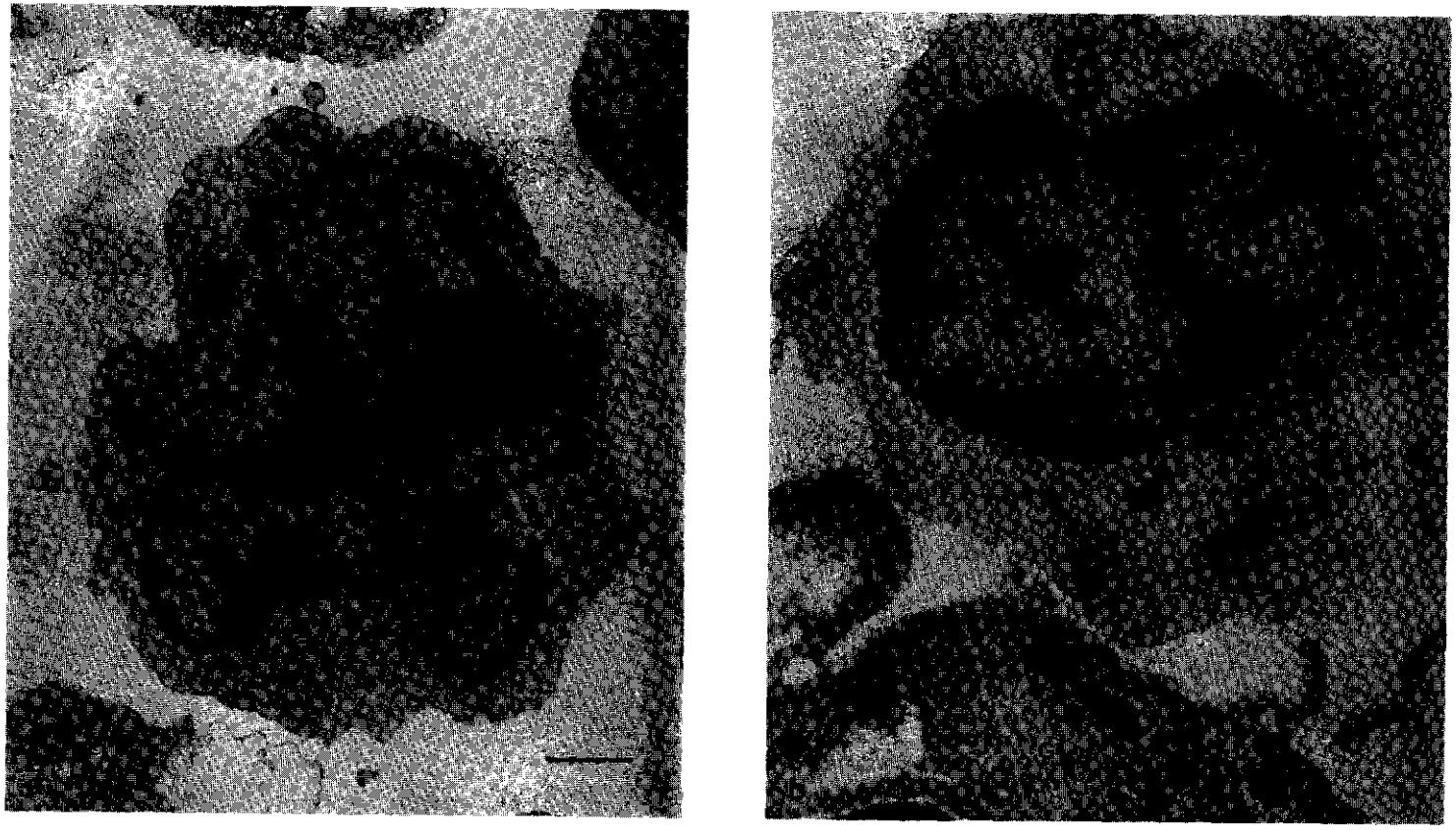

Figure 4. Electron micrographs of immunogold stained PBL. Scale bar = 0.5 μm. (a) WEI 1-immunoreactive lymphocyte. Note the absence of gold particles on an erythrocyte (E). (b) WEI 2-immunoreactive lymphocyte and part of a non-reactive granulocyte (G).

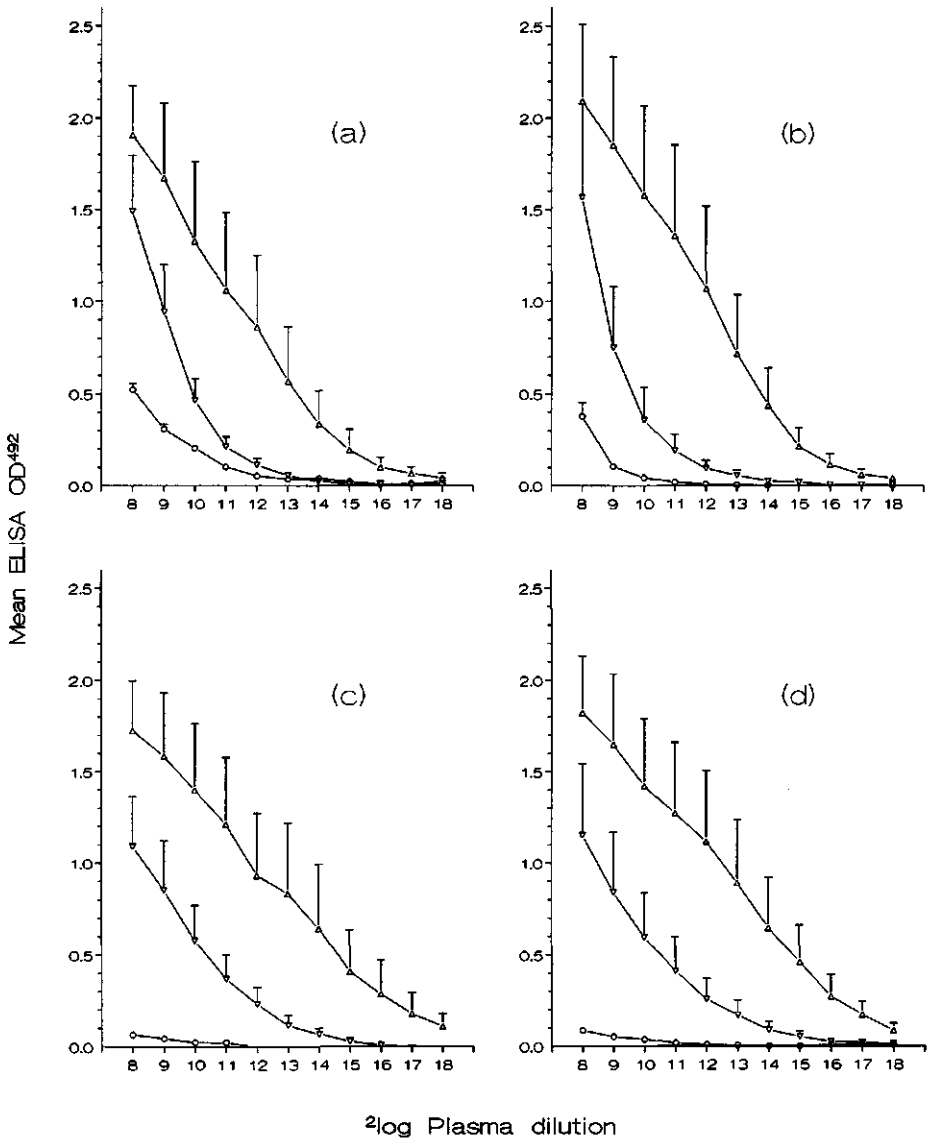

Figure 5. Mean DNP-KLH and DNP antibody titration curves after sham-immunization (—○—; n=3), priming (—▽—; n=3) and boosting (—△—; n=4). Bars represent SE. (a) DNP antibody detection using WEI 1 as secondary antibody and DNP-BSA as coating. (b) DNP antibody detection using WEI 2 as secondary antibody and DNP-BSA as coating. (c) DNP-KLH antibody detection using WEI 1 as secondary antibody and DNP-KLH as coating. (d) DNP-KLH antibody detection using WEI 2 as secondary antibody and DNP-KLH as coating.

Three forms of Ig, namely monomers of about 190 kDa, dimers of about 360 kDa and tetramers of about 760 kDa, were found in plasma of eel. All three forms are also described for serum and mucus Ig of common carp (Rombout *et al.*, 1993). However, in serum of gilthead seabream merely monomeric and tetrameric Ig were found (Navarro *et al.*, 1993). On the other hand, Lobb (1986) described dissociation of channel catfish tetrameric Ig into all possible combinations of covalently linked H-L chain units. Combining the present results with those of the authors cited, variation in structural characteristics between Ig of different teleost species appears to exist.

Ig of fish has long been thought to be composed of respective uniform H and L chains. This general concept has been abandoned because evidence for diversity of Ig in a number of teleost species has become available. Differences in molecular weights and/or antigenic determinants of H and L chains, have been described for some teleost fish (Lobb and Clem, 1981; Lobb *et al.*, 1984; Sánchez *et al.*, 1989; Koumans-van Diepen, 1993; Rombout *et al.*, 1993). The present work revealed neither indications for molecular weight differences in H and L chains, nor for the existence of antigenically different H chains. However, indirect evidence was achieved for antigenic differences within L chains. Flow cytometry showed that only about 60% of the WEI 1 (anti-H chain) reactive PBL were immunoreactive with WEI 2 (anti-L chain). This indicates that at least 2 antigenically different L chain types exist in eel. Differences in L chains have previously been described for channel catfish (Lobb *et al.*, 1984) and rainbow trout (Sánchez and Domínguez, 1991; Sánchez *et al.*, 1993).

Affinity chromatography of eel Ig, using immobilized WEI 1, revealed no detectable WEI 2-immunoreactive Ig in the fractions collected before saturation of the column. Assuming random distribution of the different L chains and considering the high sensitivity of the dot blot assay used for Ig-detection, WEI 1 seems to react with all or at least nearly all Ig.

The percentage of WEI 1-immunoreactive PBL (± 26%) appeared to be low, compared to ± 45% in rainbow trout (Thuvander *et al.*, 1990), ± 37% in channel catfish (Ainsworth *et al.*, 1990) and ± 48% in adult common carp (Koumans-van Diepen *et al.*, 1994). This discrepancy may be explained by a dilution effect of thrombocytes. About 10-60% of these cells were observed after differential leucocyte counts in cytocentrifuge preparations of the lymphocyte-enriched fraction used for determination of the relative amount of sIg^+ cells (unpublished results). The cell population used for flow cytometric analyses was gated on the basis of FSC/SSC characteristics and will consist of both

lymphocytes and thrombocytes, as size and shape of these cell types are often nearly identical (Kusuda and Ikeda, 1987). Similarity in FSC/SSC profile of lymphocytes and thrombocytes was previously reported by Koumans-van Diepen (1993) for common carp.

The respective proportions of sIg^+ lymphocytes, as determined with WEI 1, in pronephros and spleen were about 24 and 25%. These percentages are comparable with those reported for the corresponding organs in several other fish species, such as gilthead seabream (Navarro *et al.*, 1993), channel catfish (Ainsworth *et al.*, 1990), rainbow trout (DeLuca *et al.*, 1983; Thuvander *et al.*, 1990) and common carp (Koumans-van Diepen *et al.*, 1994).

Application of WEI 1 and WEI 2 in immuno-electron microscopy on isolated PBL confirmed that both mAbs actually showed a specific reaction with a part of the lymphocyte population. In accordance with the observations of Van Diepen *et al.* (1991) and Navarro *et al.* (1993) the gold particles occurred more or less clustered at the surface of the lymphocytes, suggesting that this might be a common feature for the distribution of Ig molecules on fish B cells.

Labelling intensity was consistently higher with WEI 2 than with WEI 1, in both the immunogold and immunofluorescence labelling. However, differences in staining intensity of both mAbs on fixed cells, Western blots and in the ELISA were not observed. Consequently, differences in the reactivity of the mAbs have to be attributed to the attainability of the recognized determinants on living cells rather than to differences in their affinity for Ig.

Both mAbs appeared to be suitable for the detection of anti-DNP or anti-DNP-KLH antibodies by means of an ELISA, because clear differences were not found in the titration curves after using either WEI 1 or WEI 2 as secondary antibody. The higher anti-DNP background titres found, may be dedicated to the presence of anti-BSA antibodies or antibodies cross-reacting with BSA. As a consequence, lean milk powder was used as a blocking protein, because the use of BSA caused very high background staining (not shown). In any case, clear differences were not found between the anti-DNP and anti-DNP-KLH titration curves, suggesting that eel predominantly reacts against the hapten (DNP).

In conclusion, WEI 1 and WEI 2 proved to be useful in a variety of immunoassays. They might be employed for studies on European eel health, like monitoring (functional) characteristics of the immune system and epizootic studies.

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Chapter 5

Influence of flumequine on *in vivo* mitogen responses of European eel (*Anguilla anguilla* L., 1758) lymphoid cells

M.H.T. van der Heijden[1], G.H.R. Booms[1], M.W.T. Tanck[1], J.H.W.M. Rombout[2] and J.H. Boon[1]

[1] *Department of Fish Culture and Fisheries, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

[2] *Department of Experimental Animal Morphology and Cell Biology, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

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Abstract

The influence of flumequine on mitogen induced lymphoid cell proliferation in European eels (*Anguilla anguilla* L., 1758) was studied. For this purpose an *in vivo* test, using peroral drug administration followed by successive intraperitoneal injections with concanavalin A (Con A) or bacterial lipopolysaccharide (LPS) and 5-bromo-2'-deoxyuridine, was applied. Direct counting of proliferated cells in blood smears revealed that flumequine possesses mitogenic properties. A synergistic and an antagonistic effect of the drug was observed after LPS and Con A stimulation, respectively. Flow cytometric analysis of peripheral blood lymphoid cells showed a significant reduction of the mean proportion surface immunoglobulin positive cells in the flumequine-treated animals. It is concluded that flumequine enhances proliferation of lymphoid cells (probably surface immunoglobulin negative cells) in eel under the present experimental conditions.

Key-words: European eel, Flumequine, Lymphocyte stimulation, Immunomodulation.

Introduction

Culture of European eel (*Anguilla anguilla* L., 1758) is characterized by high stocking densities of fish originating from wild populations. These fish are not selected to cope with intensive culture conditions, associated with high infection pressure from the environment and decreased resistance of the animals caused by crowding stress. These circumstances often precede infectious diseases followed by morbidity and mortality which in turn may cause high production losses. It is attempted to reduce this damage by (1) zootechnical and hygienic measures and (2), in case of failure of these measures, by administration of drugs. Vaccination, which is gaining importance in disease prevention of several cultured fish species, is not yet reported for eel culture.

At Dutch eel farms, flumequine is one of the main drugs for bacterial disease control. It is used prophylactically at times of stressful farm operations, e.g. the recurring weighing and sorting, and curatively after disease outbreaks. In this respect, the very slow elimination of flumequine in eel may be of concern (Boon *et al.*, 1991; Van der Heijden *et al.*, 1994). Several hazards have been mentioned for excessive use of drugs, like development of resistant pathogens, unacceptable residues in marketable consumption fish, pollution of the environment and last but not least (undesired) side effects on fish health (Van der Heijden *et al.*, 1992).

Some drugs have shown to interfere with immunological processes in fish (Grondel *et al.*, 1985; Dunier *et al.*, 1991; Siwicki *et al.*, 1994). Immunostimulating effects provide possibilities for boosting protective abilities of fish. For instance the anthelmintic levamisole appeared to elevate nonspecific and specific defence mechanisms, resulting in increased protection against disease in vaccination trials (Anderson and Jeney, 1992). Immunosuppression as for example described for oxytetracycline in carp (Rijkers *et al.*, 1980; Rijkers *et al.*, 1981; Grondel and Boesten, 1982; Grondel *et al.*, 1985), must be prevented because a properly functioning immune system is essential for the final elimination of a pathogen. Moreover, an impaired defence system means an increased susceptibility to (super)infections.

In the initiation of cellular and humoral responses proliferation of B- and T-lymphocytes plays an important role in mammals (Roitt *et al.*, 1993) but also in fish (Koumans-van Diepen *et al.* 1994). The extent of lymphocyte proliferation after antigenic or mitogenic stimulation may be used as a parameter to assess immunocompetence (Anderson, 1990). In the present study the effect of flumequine on

the proliferative capacity of lymphoid cells in European eel was evaluated after *in vivo* stimulation with a presumed B cell or T cell mitogen and subsequent determination of proliferated lymphoid cells in peripheral blood.

Materials and methods

Fish

Eighty non-sexed European eels (*Anguilla anguilla* L., 1758) with a mean weight (± SD) of 98.2 ± 20.9 g were obtained from a local commercial farm where the fish were raised without using any antibacterial drugs by request. Two weeks before the start of the experiment feeding was ceased and the fish were randomly assigned to eight groups of ten animals. Each group was allocated to a 70-litre aquarium with aerated, running tap water at a mean temperature (± SD) of 23 ± 0.5°C.

Experimental design

A schedule of the successive treatments per group is presented in Table 1. Manipulations of the animals were performed after sedation in water containing 300 ppm Hypnodil® (Janssen Pharmaceutica B.V., Beerse, Belgium).

Table 1. Schedule of the experimental design

Time	Treatment group							
(days)	1	2	3	4	5	6	7	8
0	Flumequine				No medication			
1	Con A	LPS	PBS	*PBS	Con A	LPS	PBS	*PBS
6	BrdU	BrdU	BrdU	PBS	BrdU	BrdU	BrdU	PBS
7	Blood sampling							

* Blood sampling for drug determination.

Drug administration

Non-medicated and medicated feed slurries were freshly prepared. The former consisted merely of ground eel feed (Provimi B.V., Rotterdam, The Netherlands) and an equal volume of distilled water whereas the latter contained also 4 $mg \cdot ml^{-1}$ flumequine (Flumiquil®, Pitman-Moore, Haarlem, The Netherlands). Each fish received 5 $ml \cdot kg^{-1}$ body weight (BW) of the allotted feed slurry by intubation of the stomach. This corresponds to a single administration of a generally used flumequine dose of 20 $mg \cdot kg^{-1}$ BW.

Mitogen and BrdU administration

The mitogens, concanavalin A (Con A) and lipopolysaccharide from *Salmonella typhimurium* (LPS), and the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Mitogen and BrdU solutions with respective concentrations of 1 and 12.5 $mg \cdot ml^{-1}$ were prepared in phosphate buffered saline (PBS; pH 7.2). Eels were intraperitoneally (i.p.) injected with Con A or LPS at a dose of 2.5 $mg \cdot kg^{-1}$ BW. At the same time, the control groups were sham treated with an equivalent volume of the solvent (PBS). One day prior to sampling, BrdU (250 $mg \cdot kg^{-1}$ BW) or an equivalent volume of PBS (controls) was i.p. injected.

Sampling

Heparinized blood samples of 0.5 ml were collected from the caudal vein. Blood smears were prepared and stored in the dark. The remaining blood was processed for flow cytometric determination of relative numbers of surface immunoglobulin positive (sIg^+) lymphoid cells.

To confirm the presence (or absence) of the drug, 0.5 ml blood of the animals in group 4 and 8 was sampled just prior to the first PBS injection (cf. Table 1). Plasma was obtained and stored at -20°C until analysis.

Immunocytochemistry

Blood smears

Air dried blood smears were fixed in methanol containing 0.03% H_2O_2 for 10 min on ice. After DNA-denaturation in 2 N HCl for 25 min at room temperature, slides were

rinsed twice in 0.05 M Tris/HCl (pH 7.6; TBS). Non-specific binding sites were blocked with 10% fetal calf serum in TBS (v/v). Subsequently, slides were successively incubated with a monoclonal anti-BrdU antibody (Eurodiagnostics B.V., Apeldoorn, The Netherlands) and with horseradish peroxidase conjugated goat anti-mouse immunoglobulin (GAM-HRP; BioRad, Richmond, CA, USA). After each incubation step the slides were rinsed 4 times for 3 min in TBS. HRP was demonstrated by a 10 min incubation with 0.1% (w/v) 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO, USA) in TBS. Smears were rinsed, counterstained with Giemsa, dehydrated and embedded in DePeX (BDH, Dorset, UK).

Per smear the percentage of proliferated ($BrdU^+$) lymphoid cells was calculated after direct counting of 200 lymphoid cells. Five randomly chosen smears of each group were analyzed accordingly.

Flow cytometry

Heparinized peripheral blood samples were diluted 1:1 with 65% RPMI-1640 (Gibco, Breda, The Netherlands) containing 10 $IU \cdot ml^{-1}$ heparin, 0.1% (w/v) NaN_3 and 1% (w/v) BSA (eRPMI; pH 7.4, 240 $mOsm \cdot kg^{-1}$). A lymphocyte enriched fraction was obtained by separating the cells over a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) density gradient (1.020 and 1.070 $g \cdot cm^{-3}$) and collecting the interphase. After washing twice in eRPMI, the cells were incubated for 30 min at 0°C with mouse monoclonal antibody (WEI 1), specifically reacting with the heavy chain of plasma and membrane bound eel immunoglobulin (Van der Heijden *et al.*, 1995). Thereafter, the cells were washed twice and incubated for another 30 min at 0°C with rabbit anti-mouse immunoglobulin conjugated to fluorescein isothiocyanaat (RAM-FITC; Dakopatts, Glostrup, Denmark). After washing, cells were analyzed with a FACStar® flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the Consort 30 data analysis package. In each sample 10 000 cells were counted and only cells complying with morphological characteristics of lymphoid cells were used to determine the relative number of sIg^+ lymphoid cells.

Determination of flumequine

Plasma samples were pooled per group and drug concentrations were determined using high performance liquid chromatography (Van der Heijden *et al.*, 1994).

Statistical analysis

Data were checked for normality and outliers by means of Wilk-Shapiro/rankit plots and box plots, respectively. Relative numbers of both $BrdU^+$ and sIg^+ cells after different treatments were examined by means of two-tailed two sample T-tests. If differences between group means were small compared with variances within groups, two-way analysis of variance (ANOVA) was used. In the latter case, subsequent pairwise comparisons of means were performed using the least significant difference method (LSD T-test). All testing was performed with the computer program STATISTIX (1985) at a significance probability level of 5%.

Results

The mean plasma flumequine level at 24 h after administration was 5.5 $\mu g \cdot ml^{-1}$, whereas the drug was absent in the sample of non-medicated animals. The proportions of both $BrdU^+$ and sIg^+ lymphoid cells were normally distributed and no outliers were detected.

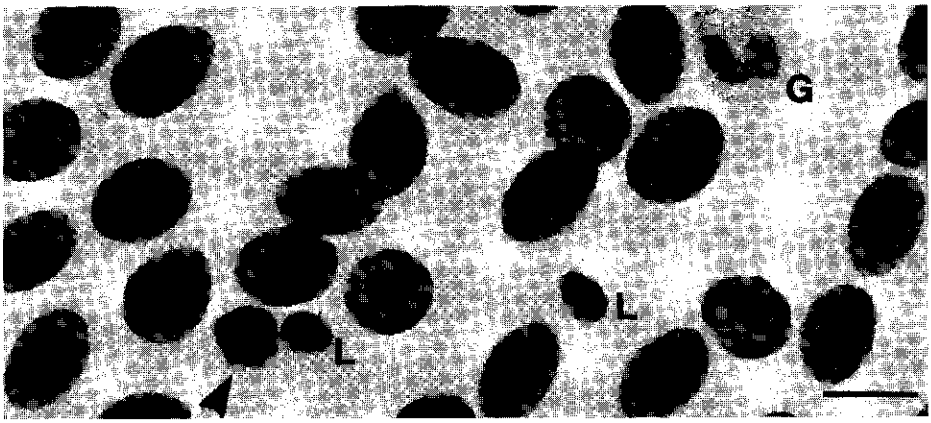

Figure 1. Micrograph of a proliferated blast-like, lymphoid cell (arrow), non-proliferated lymphoid cells (L) and a granulocyte (G) among erythrocytes in a smear of peripheral blood of European eel (*Anguilla anguilla* L., 1758), one day after BrdU treatment. Scale bar = 10 µm.

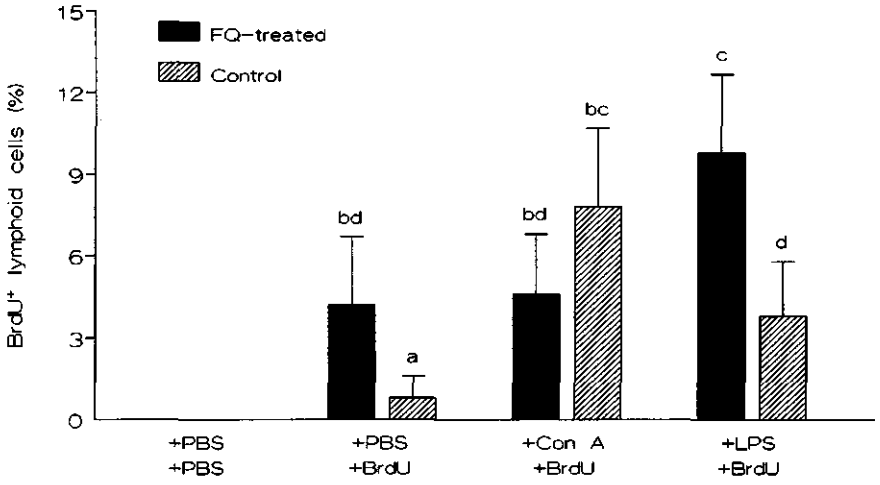

Figure 2. Lymphoid cell proliferation response, presented as mean percentages of $BrdU^+$ lymphoid cells in blood smears of flumequine (FQ)-treated and non-medicated (Control) European eel (*Anguilla anguilla* L., 1758; n = 5 per group) after *in vivo* mitogen stimulation. Bars represent standard deviation. Groups with a common superscript do not differ significantly at $P \leq 0.050$.

Incorporation of BrdU was only observed in blast-like, lymphoid cells which were brown on the slides and hence clearly distinguishable from blue, non-proliferated lymphoid cells (Figure 1). Besides, $BrdU^+$ cells were not detected in animals which did not receive BrdU. Figure 2 shows the proportions of $BrdU^+$ lymphoid cells in blood smears of flumequine-treated and non-medicated groups. Comparison of non-medicated/BrdU-treated groups revealed that both Con A (P=0.004) and LPS (P=0.027) significantly stimulated the proliferation of lymphoid cells. The mean proportion of $BrdU^+$ lymphoid cells was significantly elevated by administration of the antibacterial drug alone (P=0.035). Following Con A stimulation, the mean relative number of $BrdU^+$ lymphoid cells was not significantly different between the flumequine-treated fish and the accompanying non-medicated controls; the relative number of $BrdU^+$ cells was similar to that found in drug-treated/non-stimulated animals. The mean mitogenic response to LPS was significantly raised in the group treated with flumequine compared with both, the corresponding non-medicated group (P=0.006) and the mitogen control group (P=0.012).

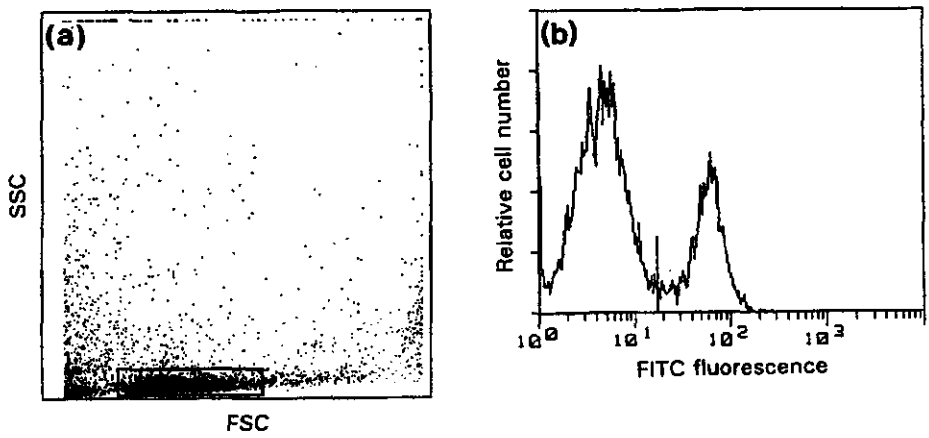

Figure 3. (a) Forward/side (90°) scatter (FSC/SSC) dot plot of a lymphocyte enriched fraction of peripheral blood cells labelled with monoclonal antibody to eel immunoglobulin. Gate contains cells regarded as lymphoid cells. (b) Fluorescence histogram of gated cells with marker position used to separate surface immunoglobulin positive and negative cells, respectively.

Table 2. Lymphoid cell proliferation response, presented as mean percentages (SD) of sIg^+ lymphoid cells in peripheral blood of flumequine (FQ)-treated and non-medicated (Control) European eel (*Anguilla anguilla* L.,1758; n=10 per group) after *in vivo* mitogen stimulation

	+Con A +BrdU	+LPS +BrdU	+PBS +BrdU	+PBS +PBS	Total mean
FQ-treated	30.74 (11.39)	33.48 (13.28)	21.68 (3.50)	24.42 (9.96)	27.57^a (11.50)
Control	31.06 (10.07)	40.31 (12.34)	27.44 (9.90)	32.85 (10.12)	32.96^b (11.83)
Total mean	30.92^{ab} (11.02)	36.90^b (13.61)	24.71^a (8.32)	28.86^a (11.19)	

Total mean values with a different superscript are significantly different at $P \leq 0.050$ within the row or column.

Figure 3 shows a representative forward/side (90°) scatter dot-plot of a lymphocyte enriched fraction of peripheral blood cells and the corresponding fluorescence histogram indicating the separation between surface immunoglobulin negative (sIg^-) and sIg^+ lymphoid cells. The mean percentages of sIg^+ lymphoid cells in peripheral blood of the different treatment groups are depicted in Table 2. Statistical analysis revealed no

significant influence of BrdU on the proportion of sIg^+ lymphoid cells. However, a significantly lower mean proportion of sIg^+ cells was observed in flumequine-treated animals compared to non-medicated controls. With regard to the combinations of mitogen and BrdU-treatment, the mean sIg^+ cell percentage appeared to be significantly higher in animals treated with LPS and BrdU than in animals which received one of the other combinations.

Discussion

The present study clearly demonstrated that both Con A and LPS induced lymphoid cell proliferation in an *in vivo* experiment with eel. The suitability of *in vivo* BrdU administration for detection of proliferating cells was previously proven in mice (Schutte *et al.*, 1987) and humans (Miller *et al.*, 1991). The absence of a significant effect of BrdU on the proportion of sIg^+ lymphoid cells in the present study indicates that the 24 h exposure to BrdU did not influence the results.

Traditionally lymphocyte proliferative capacity in fish is only measured by *in vitro* mitogenic stimulation of leucocytes (Rowley *et al.*, 1988). However, an *in vivo* study has the advantage that cell loss or damage due to a cell separating procedure is prevented. Cells remain in their natural environment providing all accessory factors for optimal proliferation. Moreover, the influence of xenobiotics on lymphocyte proliferation can be studied in compliance with the actual kinetics of the substance, including possible metabolites or degradation products.

In mammals, Con A and LPS are respectively considered to be T and B cell mitogens (Greaves and Janossy, 1972). In spite of initially conflicting reports (Warr and Simon, 1983; Caspi *et al.*, 1984), it became increasingly plausible that these mitogens have the same specificity in fish (Koumans-van Diepen *et al.*, 1994). The present results support this concept as far as LPS is concerned. The mean proportion of sIg^+ cells was significantly raised in the LPS-treated animals, indicating that this mitogen stimulates, at least predominantly, sIg^+ lymphoid cells in eel. However, the expected reduction of the mean proportion of sIg^+ cells in Con A-treated groups, as a result of the presumed T cell stimulation, could not be demonstrated.

The peroral administration of flumequine proved to be successful as shown by the determination of plasma flumequine levels. Interestingly, flumequine seems to possess

mitogenic properties because of a significant higher proportion of proliferated lymphoid cells compared with untreated animals. According to our knowledge, this phenomenon has not been reported so far. It is most probable that mainly sIg^- lymphoid cells will be involved because the proportion of sIg^+ cells tended to be lower in the flumequine-treated/non-stimulated groups. In addition, a flumequine induced shift to sIg^- cells is supported by a lower mean proportion of sIg^+ cells over all flumequine treated animals.

A synergistic effect of flumequine on the LPS response could be observed. However, the stimulating effect of flumequine on lymphoid cell proliferation was not reflected in an elevated percentage of sIg^+ cells, even not when LPS was administered. These apparently contradictory results may be explained by the results of Koumans-van Diepen *et al.* (1994) indicating that mainly dull sIg^+ cells proliferate after LPS stimulation of carp leucocytes. Probably the LPS-responding, dull sIg^+ cells are not or only partly recognized as such with the applied flow cytometric detection.

Counts of $BrdU^+$ lymphoid cells as well as the flow cytometric determination of sIg^+ cells pointed towards an antagonistic effect of flumequine on the Con A response. Although differences between individual groups were generally smaller in the determination of sIg^+ cells, an effect of flumequine on lymphoid cell proliferation was clearly demonstrated by the statistical analysis. Lymphocyte proliferation occurs in lymphoid organs whereas the response was determined in peripheral blood. Whether more pronounced changes in sIg^+ cell proportions occur in lymphoid organs remains to be investigated.

Enumeration of divided lymphoid cells by direct counting of $BrdU^+$ lymphoid cells in blood smears was rather laborious, but appeared to be a suitable method which revealed more pronounced differences between medicated and non-medicated groups than flow cytometric analysis of sIg^+ cells. However, the latter is a faster, automated method. Possibly, flow cytometric determination of incorporated BrdU, whether or not combined with simultaneous detection of sIg, as described by Koumans-van Diepen *et al.* (1994), offers prospects for combining the advantages of both methods.

Direct comparison of the present results with those of others is not possible because the immunological impact of flumequine treatment is not yet evaluated *in vivo*. However, Miyazaki *et al.* (1984), who did some toxicological examinations of other quinolones in fish, suggested interference of these drugs with DNA synthesis in rapidly proliferating cells. Whether this may be related to the alteration of mitogen responses in eel by flumequine, is not clear. Delay and inhibition of human lymphoblast

proliferation by some quinolones *in vitro*, as reported by Hussy *et al.* (1986), does not correspond with the present results.

Immunological tests, like the lymphocyte proliferation assay, give information about a particular immunological process occurring at a fixed time during an immune response. Although lymphocyte proliferation is essential for an adequate immune response, it does not provide insight in the efficacy of the defence system as a whole. An effect of drugs on a particular immunological mechanism may be more or less compensated or amplified by possible interaction with other immune functions, e.g. antigen presentation and non-specific defence systems. Therefore, from a practical point of view the overall influence of drugs on the defence system should preferably be judged after challenge experiments with relevant pathogens, under conditions which mimic actual circumstances.

From the present study, it is concluded that flumequine enhances proliferation of lymphoid cells in eel under the present experimental conditions. However, taking into account the risks for development of resistant pathogens, environmental pollution and residues in fish destined for consumption, cautious use of this drug is still recommended.

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Chapter 6

Influence of flumequine and oxytetracycline on the resistance of European eel against the parasitic swimbladder nematode *Anguillicola crassus*

M.H.T. van der Heijden[1], G.M. Helders[2], G.H.R. Booms[1], E.A Huisman[1], J.H.W.M. Rombout[2] and J.H. Boon[1]

[1] *Department of Fish Culture and Fisheries, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

[2] *Department of Experimental Animal Morphology and Cell Biology, Wageningen Agricultural University, P.O.Box 338, 6700 AH Wageningen, The Netherlands*

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Abstract

The effect of the antibacterial drugs flumequine and oxytetracycline on the defence system of European eel (*Anguilla anguilla* L.,1758) was investigated using an experimentally induced infection with the parasitic swimbladder nematode *Anguillicola crassus*. Eight weeks after oral administration of infective larvae, the mean recovery of the parasites in flumequine-treated eels was lower than in the non-medicated control animals and significantly lower than in oxytetracycline-treated eels. Mean numbers of peripheral blood granulocytes and B-lymphocytes as well as the total amount of circulating lymphoid cells showed an increase as a result of the infection, while drug treatment significantly affected the quantities of the lymphoid cells. The difference in protection against the parasite after flumequine or oxytetracycline administration points towards a modulation of the fish resistance as a result of the drug treatment. The results favour a modulation of the cellular rather than the humoral response as no specific antibodies were found.

Key-words: Antibacterial drugs, European eel, Resistance, *Anguillicola crassus*, Leucocytes.

Introduction

The antibacterial drugs flumequine and oxytetracycline are commonly used to control bacterial diseases in fish culture. Several studies have shown the interference of these drugs with the immune system of fish (Rijkers *et al.*, 1980; Rijkers *et al.*, 1981; Grondel *et al.*, 1982; Grondel *et al.*, 1985; Grondel *et al.*, 1987; Van der Heijden *et al.*, 1995a). The latter authors report effects on several immunological processes, such as lymphocyte proliferation, antibody production and graft rejection. However, none of these studies deals with the actual impact of the drugs on the resistance against infectious diseases.

In European eel, both flumequine and oxytetracycline are eliminated very slowly compared to other teleost species (Nouws *et al.*, 1993; Van der Heijden *et al.*, 1994). Consequently, eel is an interesting species for studying side effects of these drugs. To evaluate immunomodulating properties of drugs, parasitic infection models are very useful, because resistance against parasites is based on a series of, often mutually dependent, protective mechanisms (Wakelin, 1984; Roitt *et al.*, 1993), which can thus be approached in an integrated way. A parasitic infection model for eel, using *Anguillicola crassus*, a commonly occurring, sanguivorous nematode residing in the swimbladder, is already available (Haenen *et al.*, 1989; Boon *et al.*, 1990a; Boon *et al.*, 1990b; De Charleroy *et al.*, 1990a). This *A. crassus* model may be suitable for the intended challenge test since there is no evidence for any susceptibility of these nematodes to antibacterial drugs. Moreover, next to the non-specific reactions described by Molnár *et al.* (1993), data are available for the existence of protective immunity to the establishment and development of *A. crassus* in eel. Boon *et al.* (1990a) demonstrated a retarded development of the nematode larvae in the host after repeated experimental infections with low numbers of infective larvae of *A. crassus*. Furthermore, both the prevalence of the parasite and the individual nematode burden in large wild caught eels from Dutch fresh water lakes have decreased since 1988, whereas this trend was not observed in small eels during the same observation period (Haenen *et al.*, 1994a). In addition, specific humoral antibodies to the parasite in naturally infected eels were described by Buchmann *et al.* (1991) and Höglund and Pilström (1994).

The present report describes an evaluation of flumequine and oxytetracycline treatment of eel with regard to the resistance against an artificially induced infection with *A. crassus*, assessed by determination of the recovery of parasites in the

swimbladder, 8 weeks after inoculation. Moreover, during the experiment, *A. crassus* specific antibodies and numbers of granulocytes, lymphoid cells and B-lymphocytes in peripheral blood of the host were determined to estimate the activity of the immune system.

Materials and methods

Fish

A stock of 100 non-sexed European eels (*Anguilla anguilla* L., 1758), originating from an *A. crassus*-free eel farm, were kept in the hatchery of 'De Haar - vissen' at the Wageningen Agricultural University for about 6 months without using antibacterial drugs. Fourteen days prior to the start of the experiment, 80 fish (mean weight ± SD, 72.6 ± 13.5 g), randomly selected from the stock, were divided into 8 groups of equal size. Each group was transferred to a 70-litre aquarium, supplied with non-recirculating, aerated tap water of 22.5 ± 1°C (mean ± SD) at a flow rate of about 70 $l \cdot h^{-1}$, for adaptation. During the adaptation and experimental period fish were starved.

Infective larvae

Infective third stage larvae (L_3) of *Anguillicola crassus* Kuwahara, Niimi et Itagaki, 1974 (Moravec and Taraschewski, 1988) were produced according to the method described by Haenen *et al.* (1994b). Briefly, L_3 were cultured by feeding second stage larvae, isolated from wild caught eels, to the intermediate hosts (cyclopoid copepods). After 2 weeks, L_3 were harvested by crushing the copepods with a tissue potter. Subsequently, the released L_3 were extracted from the pottered suspension by sedimentation in a Baermann funnel. Sixty doses of 12 larvae with 0.5 ml RPMI-1640 medium (Gibco, Breda, The Netherlands) were prepared in tubes and stored at 4°C until the next day. Before use the larval suspensions were substantially shaken after being put at room temperature for 2 h.

Experimental design

Each of 4 different treatments was randomly assigned to 2 groups of 10 fish. Two of the treatments comprehended administration of flumequine or oxytetracycline respectively, followed by infection with *A. crassus*. Controls were either only infected with the parasite or they were neither medicated nor infected.

Drug administration and infection technique

Flumequine (Flumiquil®; Pitman-Moore, Haarlem, The Netherlands) or oxytetracycline (oxytetracycline-HCl; A.U.V., Cuijk, The Netherlands), dissolved in physiological salt solution (A.U.V. Cuijk, The Netherlands), were administered by injection into the epaxial trunk musculature (i.m.) on both sides of the animal at a dose of 10 and 60 $mg \cdot kg^{-1}$ body weight (BW), respectively. Control animals were sham-treated by i.m. injection of an equivalent volume (2 $ml \cdot kg^{-1}$ BW) of the solvent.

Directly after the injection, each fish was perorally inoculated with either one of the previously prepared doses of 12 L_3 in RPMI-1640 or merely the medium in case of controls. This was done by means of a stomach tube with an attached 1 ml disposable syringe. Afterwards, the tubes in which the larvae had been stored were checked for possibly remaining larvae to correct the inoculated dose.

To facilitate the manipulations during injection and infection as well as to prevent regurgitation of larvae, fish were profoundly sedated by bathing in 15 ppm metomidate (Hypnodil®, Janssen Parmaceutica B.V., Beerse, Belgium) for about 20 minutes.

Sampling

From 2 until 6 weeks post infection (p.i.), heparinized blood samples of 0.5 ml were collected weekly from each eel by puncturing the caudal vein after sedation (5 min in 15 ppm metomidate). After preparing blood smears, the samples were centrifuged (300×*g*, 10 min at 4°C) and plasma was stored at -20°C. The buffy coat was collected and stored on ice until further processing.

At 8 weeks p.i., eels were bled and plasma was obtained and stored as described above. Subsequently, the animals were euthanized by decapitation, the swimbladders were extirpated and examined for the numbers and developmental stages (Boon *et al.*, 1990a) of parasites present in the lumen or wall.

Peripheral blood leucocytes (PBL)

Blood smears

The air dried blood smears were fixed in methanol for 5 min, stained according to the May-Grünwald-Giemsa method (Romeis, 1968), dehydrated and embedded in DePeX (BDH, Dorset, UK). The relative abundance of the different white blood cell types (Boon *et al.*, 1990b) was determined after differential counting of a total of 100 leucocytes per smear at a magnification of 1000×.

Flow cytometry

The collected buffy coat cells were purified by centrifugation over Lymphoprep® (Nycomed, Oslo, Norway) for 30 min at 1500×*g* and 4°C. PBL harvested from the interface were washed twice with eRPMI (65% RPMI-1640 + 10 $IU \cdot ml^{-1}$ heparin + 0.1% (w/v) NaN_3 + 1% (w/v) BSA; pH 7.4, 240 $mOsm \cdot kg^{-1}$) by centrifuging for 10 min at 680×*g* and 4°C. Subsequently, the isolated PBL were resuspended in 1 ml eRPMI, counted by means of a Coulter Counter® (model ZM; Coulter Electronics Ltd, Luton, UK) and diluted to a density of 2×10^6 $cells \cdot ml^{-1}$. The percentage of surface immunoglobulin positive lymphoid cells (B cells) was determined with a FACStar® flow cytometer (Becton Dickinson, Mountain View, CA, USA), using a mouse monoclonal antibody specific for eel immunoglobulin (Ig) heavy chain (WEI 1; 1:400; Van der Heijden *et al.*, 1995b) and fluorescein isothiocyanate conjugated rabbit anti-mouse Ig (RAM-FITC; 1:100; Dakopatts, Glostrup, Denmark) as second antibody. Lymphoid cells were gated and analyzed for labelling using the Consort 30 data analysis package.

The absolute number of cells in PBL subpopulations was estimated by combining the data obtained by Coulter Counter®, FACStar® and differential leucocyte counts.

ELISA for detection of antibodies to <u>A. crassus</u>

Plasma samples collected at 3 weeks p.i. were used individually, whereas those from 2, 4, 5, 6 and 8 weeks p.i. were pooled per group. Screening for *A. crassus*-specific antibodies was done using an ELISA according to Höglund and Pilström (1994) with some modifications. In summary, high affinity microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with whole worm extract purified by isoelectric focusing and post-coated with 5% (w/v) lean milk powder in 0.5% (v/v) Tween 20-

containing phosphate buffered saline. After washing, plates were successively incubated with serial dilutions of plasma samples, a mouse monoclonal antibody specific for eel Ig light chain (WEI 2; 1:200; Van der Heijden *et al.*, 1995b) and rabbit anti-mouse Ig conjugated with horseradish peroxidase (RAM-HRP; 1:5000; Dakopatts, Glostrup, Denmark). HRP was demonstrated using a substrate solution consisting of tetramethylbenzidine and H_2O_2 in acetate/citrate buffer. After 10 min, the reaction was stopped with 0.5 M sulphuric acid. The resulting absorbence was recorded at 450 nm in an Easyreader spectrophotometer (SLT-labinstruments, Vienna, Austria). To correct for plate differences each plate contained the positive and negative control sera described by Höglund and Pilström (1994).

Determination of antibacterial drugs

Plasma samples collected at 2 weeks p.i. were pooled per group and checked for the presence or absence of flumequine and oxytetracycline by column-switching high performance liquid chromatography. The flumequine determination was carried out according to the method described by Van der Heijden *et al.* (1994). Oxytetracycline was determined after concentration from diluted plasma using a column packed with Sep-Pak C-18 material (Waters, Millford, MA, USA) and subsequent separation on an Inertsil ODS-2 analytical column (Chrompack, Bergen op Zoom, The Netherlands). UV detection was performed at 365 nm.

Statistical analysis

Data are presented as mean values ± standard error of the mean (SE). The numbers of *A. crassus* retrieved, the recovery (number of parasites retrieved divided by corrected number of L_3 administered) and the occurrence of different developmental stages of the parasite in the infected groups were examined by analysis of variance (ANOVA), using the factors fish, group, treatment and their interactions. Data on estimated numbers of granulocytes, lymphoid cells and B cells were first tested for outliers by means of box plots. Thereafter, the remaining data were subjected to ANOVA across weeks (factor time in addition to factors mentioned above) and ANOVA within weeks (model as above). Where possible the models were reduced by omitting non-significant interaction terms. After ANOVA, the least significant difference method (LSD-T test) was

employed for pairwise comparison of means. STATISTIX (1985) software was used for all statistical analyses and differences were considered to be significant at a probability of 0.050 or less.

Results

Medicated groups were positive for the injected flumequine or oxytetracycline. At 2 weeks p.i., the respective mean drug concentrations in plasma of those animals were approximately 0.2 and 10 $\mu g \cdot ml^{-1}$, while neither of both drugs could be detected in the non-medicated groups.

The number of *A. crassus* retrieved at the end of the experiment in the flumequine-treated fish (1.2 ± 0.5) was lower than in the non-medicated controls (2.2 ± 0.5) and appeared to be significantly lower than in the oxytetracycline-treated animals (2.9 ± 0.6). Swimbladders of sham-infected eels contained neither larval nor adult specimens of the parasite. The corrected mean doses of L_3 were 11.8 for the medicated groups and 11.4 for the infected, non-medicated groups. This lead to recoveries as shown in Figure 1. Flumequine treatment resulted in a 48.8% reduction of the recovery relative to the controls and a significant 60.6% reduction compared to oxytetracycline treatment. The proportion of different developmental stages (L_3, fourth stage larvae, pre-adult or adult) in the recovered *A. crassus* population per treatment is presented in Table 1. ANOVA did not reveal an influence of the drugs on the development of the parasite larvae. L_3 were not found at all, whereas adult parasites always comprised about 70 to 80% of the worm burden.

Figure 2 shows the estimated numbers of granulocytes, lymphoid cells and B-lymphocytes in peripheral blood of the non-medicated groups during the experiment. The influence of the parasite infection was clear because the mean counts of the different leucocyte types were consistently higher in the infected animals. Comparison across weeks showed a significant influence of the infection on lymphoid cell and B-lymphocyte numbers. Furthermore, there was a significant time effect on all parameters. In week 3, 4 and 6 p.i. the mean numbers of lymphoid cells were significantly higher in infected eels, while the granulocyte and B cell counts of these fish were significantly higher at 3 and 4 weeks p.i., respectively.

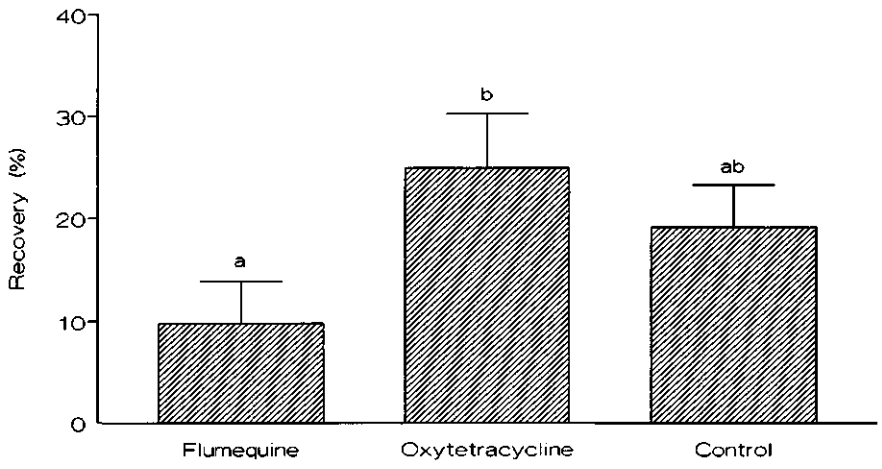

Figure 1. Mean recovery percentage of inoculated *Anguillicola crassus* in flumequine or oxytetracycline treated and non-medicated (Control) European eels (*Anguilla anguilla* L., 1758; n=20 per treatment) at the end of the experiment. Bars represent the standard error of the mean. Groups with a superscript in common do not differ significantly at P≤0.050.

Table 1. Mean percentage (± SE) of the different developmental stages of *Anguillicola crassus* in relation to the total worm burden for the various treatments at the end of the experiment

Treatment*		Developmental stage			
Drug	Inf.	L_3	L_4	Pre-adult	Adult
FQ	+	0.0 ± 0.0	0.0 ± 0.0	21.7 ± 11.7	78.3 ± 11.7
OTC	+	0.0 ± 0.0	7.1 ± 4.0	20.0 ± 14.4	72.9 ± 10.4
-	+	0.0 ± 0.0	3.7 ± 3.7	27.8 ± 5.6	68.5 ± 1.9
-	-	NP	NP	NP	NP

* n=20 per treatment. Abbreviations: Inf., infection with *A. crassus*; L_3, third stage larvae; L_4, fourth stage larvae; FQ, flumequine; OTC, oxytetracycline; NP, not present.

The estimated number of cells in the aforementioned PBL subpopulations for the infected groups is depicted in Figure 3. Once more, there appeared to be a significant influence of time on each of these parameters, but an overall treatment effect was not found. However, at 3 weeks p.i. the mean numbers of both lymphoid cells and B-

lymphocytes were significantly raised in oxytetracycline-treated animals in comparison with those treated with flumequine.

Specific antibodies to the parasite were not demonstrated in any of the plasma samples analyzed. All samples showed ELISA optical densities corresponding with the negative control sera, while positive control sera showed a clear reaction.

Discussion

Plasma drug concentrations measured in the present experiment at 2 weeks after injection are in accordance with those reported in previous studies on the kinetics of flumequine (Van der Heijden *et al.*, 1994) and oxytetracycline (Nouws *et al.*, 1993) in eel. This means that demonstrable concentrations of the drugs will be present in the eel tissues for at least 4 to 6 weeks after administration. So, a possible influence of the drugs on the defence mechanisms may be assumed during the same period. Moreover, this time span coincides with the expected residence of L_3 in the tissues and their migration from the lumen of the digestive tract to the swimbladder (Haenen *et al.*, 1989; De Charleroy *et al.* 1990b). The resulting most intimate contact between the host immune system and the parasites will be the major stimulus for resistance. Therefore, the arrival of *A. crassus* in the swimbladder will be dependent on the condition and functioning of the (compromised) defence system during the larval migration phase. This means that the recovery of the nematodes, as recorded in this study, provides an indicator for drug associated immunomodulation.

The percentage of worms retrieved in swimbladders of non-medicated eels was approximately 20% of the dose administered, which is somewhat lower than the 25% found by Boon *et al.* (1990a) and the 38% found by De Charleroy *et al.* (1990a). However, the larvae they used for infection were taken from infected glass eel and carp, respectively. As a consequence, these L_3 were more or less selected for viability because they had proven to be able to penetrate the tissues in an intermediate or final host.

The recovery of the worms in the present experiment ranged from about 10% in flumequine-treated eels to almost 25% in the oxytetracycline-treated animals. The higher recovery in the latter group compared to the controls may be explained by the immunosuppressive effects attributed to oxytetracycline (Rijkers *et al.*, 1980; Rijkers *et al.*, 1981; Grondel *et al.*, 1982; Grondel *et al.*, 1985; Grondel *et al.*, 1987). On the other

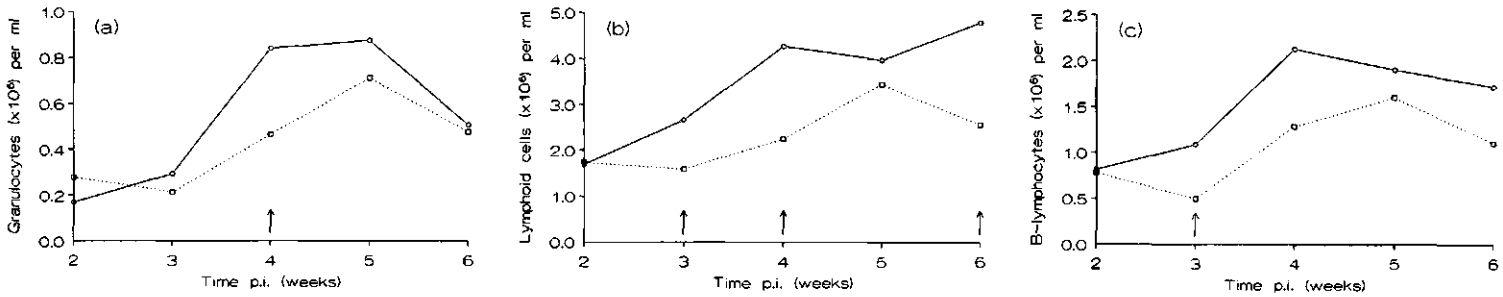

Figure 2. Number of granulocytes (a), lymphoid cells (b) and B-lymphocytes (c) per ml peripheral blood of *Anguillicola crassus*-infected (— o —) and sham-infected (····□····) European eels (*Anguilla anguilla* L., 1758) from 2 until 6 weeks after infection (p.i.). Each point represents the arithmic mean of a group of 20 fish and ↑ points to a significant difference between the values (P≤0.050).

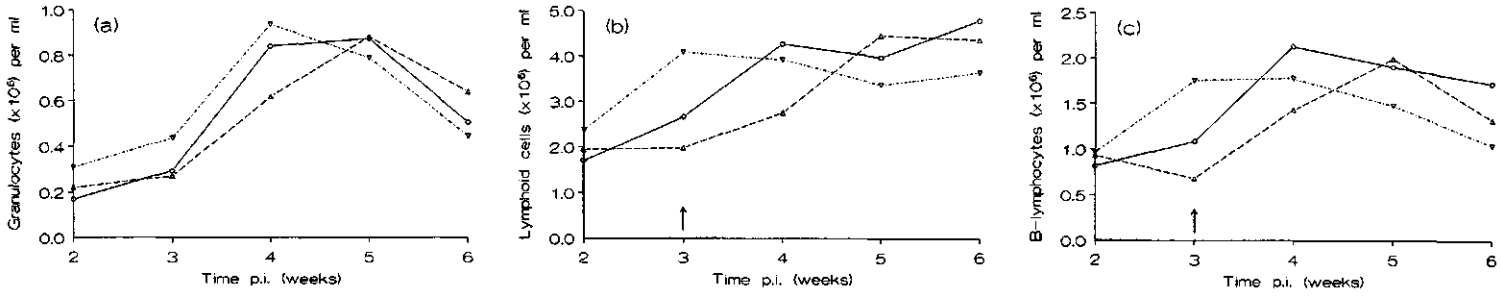

Figure 3. Number of granulocytes (a), lymphoid cells (b) and B-lymphocytes (c) per ml peripheral blood of *Anguillicola crassus*-infected European eels (*Anguilla anguilla* L., 1758) treated with flumequine (– –▵– –) or oxytetracycline (– ·▽·– ·) and non-medicated controls (— o —) from 2 until 6 weeks after infection (p.i.). Each point represents the arithmic mean of a group of 20 fish and ↑ points to a significant difference between the medicated groups (P≤0.050).

hand, there are indications that flumequine enhances proliferation of surface immunoglobulin negative (sIg^-) lymphoid cells (Van der Heijden *et al.*, 1995a). This may account for the lower nematode recovery in the flumequine treated fish, the more so as T-lymphocytes and natural killer cells, both sIg^- lymphoid cells, are assumed to fulfil a key role in the immunological responses against most parasitic pathogens (Wakelin, 1984; Woo, 1992; Roitt *et al.*, 1993).

Due to large variation in worm burden within groups, the difference between parasite recoveries in the drug-treated animals and the controls was not significant in spite of considerable differences in the mean recovery. As resistance against parasitic infections of both mammals (Wakelin, 1985) and fish (Bower and Margolis, 1984; Wiegertjes *et al.*, 1995) appears to be dependent on the genetic constitution of the host, the individual variation in worm burden in the present study might be attributed to assumed large genetic variation within eel populations.

Activation of the eel immune system as a result of the presence of *A. crassus* in the tissues was illustrated by increased numbers of circulating leucocytes. This quantitatively substantiates the previously reported influence of *A. crassus* infection on the relative abundance of these cells (Boon *et al.*, 1990b). However, a time effect on PBL numbers during the experiment could be observed for infected as well as for non-infected animals. This probably indicates that a certain degree of stress, caused by the experimental procedure, has influenced the leucocyte counts as well (Pickering, 1986).

Comparison of the drug treatments showed a significant difference in lymphoid cell and B-lymphocyte counts at 3 weeks p.i. These PBL counts obviously correlated with the *A. crassus* recovery data ($r=0.908$ and 0.840, respectively; $P<0.050$), suggesting that the numbers of lymphoid cells are not only influenced by the drugs but possibly also by the worm load. However, the recovery was determined 8 weeks p.i. and mainly consisted of adult and pre-adult stages retrieved from the swimbladder lumen, which may be considered an immune privileged site because of the remoteness from any of the immediate effectors of resistance. Nevertheless, a higher recovery will have been preceded by a higher number of active, tissue-dwelling larvae, leading to an increased reaction of the immune system.

Specific antibodies to *A. crassus* were not found in the present study. This seems to be in contrast with the results of Buchmann *et al.* (1991) and Höglund and Pilström (1994) who demonstrated anti-*A. crassus* antibodies in serum of naturally infected fish or fish intraperitoneally injected with adult whole-worm extract. However, the amount of antigen

in their respective studies was much higher than in the present case, where a single oral infection with about 12 L_3 was used. This dose might be too low to elicit a measurable primary antibody response, all the more because it can not be excluded that part of the L_3 administered did not penetrate the digestive tract epithelium. Conversely, assuming that all inoculated L_3 actually entered the eel tissues and taking into account that only about 10 to 25% was recovered from the swimbladder, there are still around 10 larvae left. These larvae would constitute a considerable antigen source for the host, especially after their death and subsequent disintegration. If this is the case, the absence of specific antibodies should probably be explained by development of latent or encapsulated larvae (Molnár, 1994), which will be less immunogenic. Furthermore, the feed deprivation of the experimental animals during the present study, but also stress caused by this or otherwise, may have played a role by suppression of antibody production (Henken *et al.*, 1987; Ellis, 1981).

Meyer (1989) suggested a broad spectrum activity of quinolones (e.g. flumequine) against bacteria, as well as fungi, protozoans and helminths without providing any evidence. His statement might be based on immunostimulation by these drugs, as a direct effect on such a broad spectrum of pathogens, without severe adverse effects on the target animal, is very improbable. Although the present experimental design does not exclude a direct influence of the drugs on the nematodes, it is more likely that the difference in protection against *A. crassus* in flumequine- and oxytetracycline-treated eels is due to a modulation of the fish resistance by these drugs. In this respect it is worthwhile to mention that the mean number of white spots, on flumequine-, furazolidone- and sham-treated groups of eels, after an accidental infection with the protozoan parasite *Ichthyophthirius multifiliis* in our laboratory, appeared to be significantly lower on flumequine-treated animals (unpublished results).

In conclusion, a better protection against *A. crassus* is achieved in eels treated with flumequine compared to those treated with oxytetracycline. This difference seems to be related to an influence of these drugs on the fish defence system. The present results point to a modulation of the cellular rather than the humoral response as no specific antibodies against the nematode could be detected.

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Chapter 7

General discussion

Contents

Introduction

A disproportionate increase in the use of antibacterial drugs in relation to production, as came along with the expansion of the intensive fish culture (chapter 1), took previously place after the introduction of intensive farming techniques in traditional animal husbandry. Considering the promising developments in the latter industry (Van den Bogaard *et al.*, 1994), the phenomenon should probably be regarded as an initial attempt to restore the disrupted balance between host and pathogen. This imbalance results from both decreased resistance of the intensively farmed animals and ready transmission of pathogens in the densely stocked populations. These latter factors which combine to favour disease are also factors which hinder easy control of disease. Nevertheless, the increasing need for sustainable production methods asks for emphasis on disease prevention rather than on treatment as the primary focus of health management. Simply increasing the use of antibacterial drugs does not really solve the problems associated with bacterial diseases; it may even mask deficient farm management while additionally carrying a significant risk for the consumer, the environment and the target animals themselves (chapter 1). Therefore, attention should be paid to a strict hygiene and improvement of the general health condition of the animals through further optimization of husbandry techniques along with application of appropriate monitoring systems. Moreover, application of vaccines and/or immunostimulants may also contribute to prevent disease related production losses.

Preventive measures as mentioned above, could reduce outbreaks of bacterial diseases in aquaculture and thus the need for antibacterial drugs. However, if prevention is not effective, the use of antibacterial drugs is a last resort, and as such these drugs form an indispensable part of fish health care. Unfortunately, in most fish farming countries the number of drugs registered for use in fish is not in proportion to the production volume of the sector and usually insufficient to warrant an optimal disease treatment. In some countries, including The Netherlands, even no drugs are fully approved for use in food fish (Anonymous, 1992). The resulting, inevitable off-label or unauthorized use of drugs constitutes an unmistakable problem for the aquaculture industry through a lack of necessary specific knowledge. Next to increasing concerns about antibacterial drug resistance, potential effects of drug residues in food and environmental pollution, little is actually known about the impact of drugs on the health and associated disease resistance of treated animals. This also applies to the use of

flumequine in Dutch eel culture, which is the common theme of the experiments presented in this thesis.

In the current chapter the main results of the constituent papers of this thesis will be briefly summarized and discussed in a broader context. Attention will be paid to matters that were previously underexposed and to the implications of some aspects for fish culture in general and the culture of European eel in particular. Moreover, some recommendations for further research will be made. The respective chapters should be consulted for complete results with accompanying more detailed discussions.

On the kinetics of flumequine

Pharmacokinetic data are indispensable to estimate the clinical efficacy of a drug as well as its possible negative effects. Accordingly, the series of experiments in the present thesis opens with a couple of studies on the disposition kinetics of flumequine, a drug commonly used in intensive aquaculture production systems and the central drug in further experiments.

Interspecies differences with regard to the plasma disposition after a single peroral administration of flumequine were studied in common carp, African catfish and European eel. All species showed an unexpectedly rapid absorption after peroral administration of the drug, which lead to a lack of data for a proper characterization of the absorption phase. This might be connected with the fact that the drug was presented in dissolved form, which might have influenced the rate of absorption. In contrast, intramuscular administration of a flumequine suspension to European eels resulted in a much slower absorption (chapter 3; Boon *et al.*, 1991). During the subsequent distribution and elimination phases the plasma flumequine concentrations of the individual fish fitted well into a two-compartment open pharmacokinetic model. However, statistically significant interspecies differences were observed for most of the determined pharmacokinetic parameters concerning these phases. Moreover, the results demonstrated an extremely slow elimination of flumequine in European eels.

It went beyond the scope of this thesis to find an explanation for this most intriguing slow elimination of flumequine in eels. However, several reasons can be put forward which may contribute to this phenomenon.

(1) On average eels have an exceptionally high body fat content (Heinsbroek *et al.*,

1989; Garcia-Gallego *et al.*, 1993; Heinsbroek *et al.*, 1993). Considerable quantities of substances with high lipid solubility - like flumequine - may be stored in this fat (De Boer and Pieters, 1991). Once assimilated in fat, these substances are usually slowly released from this depot, which is partly due to the poor vascularization of this tissue. However, this theory was not supported by tissue analyses (chapter 3). Despite the highest flumequine concentrations were initially found in fat tissue, a comparatively rapid decline was observed. In addition, there were no indications for accumulation of flumequine in any other tissue examined. However, good tissue distribution and the slow release from tissues, especially bone and skin, will play a role in the slow elimination from plasma.

(2) Almost no biotransformation of flumequine was observed in eel nor in carp and catfish (chapter 2). This agrees with the results obtained by Sohlberg *et al.* (1990), who did not find any metabolites of flumequine after oral and intra-arterial administration of the drug to rainbow trout. Moreover, after intramuscular injection of flumequine in eel more than 85% of the dose administered was recovered in the holding water in unchanged form (Boon *et al.*, 1991). Nevertheless, several flumequine metabolites (7-hydroxy-flumequine and glucuronide conjugates) have been reported in mammals (Mevius, 1990). In principle, the same metabolites should be expected in fish because the current knowledge confirms the existence of xenobiotic metabolizing enzyme systems in these animals with striking similarities with their mammalian analogues (Guarino, 1986).

(3) Possible absence of active renal (tubular) excretion of flumequine in eels, as previously suggested by Boon *et al.* (1991). The relative importance of passive excretion processes in the latter study was supported by a strong correlation between the pattern of the plasma concentration and the excretion ($\mu g \cdot kg^{-1} \cdot h^{-1}$) of the drug. Generally, renal excretion is assumed to be of minor importance for the elimination of drugs in fish. This is believed to mainly depend on diffusion from the capillaries in gills (and possibly skin) into the surrounding water and on hepatic excretion (Hickman and Trump, 1969). The gill surface of eels is comparatively small as opposed to other fish species (Byczkowska-Smyk, 1958), which may play a role in the extremely slow elimination of flumequine in this species.

(4) Entero-hepatic recirculation, which is reported for several quinolone drugs in mammals (Schentag *et al.*, 1990). Although there were no direct indications for biliary excretion of flumequine in eels nor for a subsequent reabsorption from the intestinal

tract in the studies performed, the long persistence of the drug may well be related to this phenomenon, because of the rapid flumequine absorption from the gastro-intestinal tract and the relatively high concentrations of this drug found in liver tissue (chapter 3).

Considering that the afore mentioned aspects may play a role in the observed long elimination half-life of flumequine in eels, further research to elucidate the underlying causes is highly recommended.

On the impact of flumequine

Health parameters

It is difficult to quantify the health condition of an animal. However, since many cases arise in which some degree of health assessment is desired, several authors made an effort to define parameters that can be used to describe the physical well-being of animals (Blaxhall, 1972; Miller *et al.*, 1983; Wedemeyer *et al.*, 1983; Pickering and Pottinger, 1988; Anderson, 1990; Goede and Barton, 1990; Novotny and Beeman, 1990; Schreck, 1990; Iwama *et al.*, 1995). So far, parameters related to various disciplines (e.g. ethology, endocrinology, haematology, immunology) as well as interdisciplinary approaches have been advocated. In the present thesis research was focused on the functioning of the defence system and associated disease resistance, because antibacterial drugs are known as potential immunomodulators (Grondel and van Muiswinkel, 1986; Descotes, 1988). Moreover, some of these drugs proved to be very slowly eliminated by European eels (chapters 1, 2, 3), which may increase the significance of any side effects in this particular species.

To evaluate the possible interference of flumequine with the defence system of European eel, essentially three different approaches were used in the studies presented. A shift in the ratio or numbers of different peripheral blood leucocytes was assumed to point towards a modulation of the defence system. In this respect, special attention was given to the relative abundance of surface immunoglobulin positive (B) lymphocytes. Furthermore, the proliferative capacity of lymphocytes was assessed by means of a lymphocyte proliferation assay using a presumed B cell (lipopolysaccharide; LPS) or T cell (concanavalin A; Con A) mitogen (Roitt *et al.*, 1993). Finally, an experimental infection with the parasitic swimbladder nematode *Anguillicola crassus* was performed

to address the impact of the drug on the total resistance of the exposed host.

Partially the necessary tools yet had to be developed. To this end, monoclonal antibodies (mAbs) to immunoglobulin (Ig) of European eel were produced (chapter 4). The selected mAbs, designated WEI 1 and WEI 2, proved to be specifically reactive to the Ig heavy and light chain, respectively. Testing of the reaction pattern using various immuno(cyto)chemical techniques revealed that WEI 1 probably recognizes all Ig molecules, whereas WEI 2 reacts with part of them. Nevertheless, using either of the mAbs, similar titres were achieved in enzyme-linked immunosorbent assays (ELISAs) for detection of plasma Ig. Consequently, a possible difference in background should be decisive in the selection of the most appropriate mAb for this application. With regard to immunocytochemistry, however, there appeared to be a clear difference in the percentage of cells labelled by WEI 1 or by WEI 2. Obviously, WEI 1 was considered to be the most suitable reagent for routine monitoring, most if not all, Ig-bearing (B) lymphocytes.

Flow cytometric determination of B-lymphocyte proportions in peripheral blood turned out to be an adequate method for preliminary establishment of immune alterations in fish (chapters 5, 6). The procedure is comparatively fast, so the rather large variation in this parameter can easily be countered by adapting the sample size accordingly. Moreover, since the animals do not have to be sacrificed, there is a possibility to follow the developments in individual animals over time. However, although a shift in the proportion of B-lymphocytes (and hence in the complementary fraction of Ig negative lymphoid cells) definitely reflects a change in immune status, additional tests are required to assess the possible positive or negative influence on the animals health.

The proliferative capacity of lymphocytes is generally measured by means of *in vitro* assays using stimulation with mitogens or allogeneic leucocytes and incorporation of tritium thymidine into DNA. Although these tests are very sensitive several disadvantages were recognized. Specific laboratory equipment suitable for the use of isotopes is needed and, more important, the cells are cultured in a highly unnatural environment, which may hamper the interpretation of the results. Therefore, preference was given to development of a more realistic, *in vivo* counterpart of the mitogen mediated lymphocyte proliferation assay (chapter 5). Essentially, this test includes intraperitoneal injections of mitogen and 5-bromo-2'-deoxyuridine (BrdU). The latter is incorporated into DNA of dividing cells, which are enumerated in blood smears after

immunocytochemical detection of BrdU. All in all this *in vivo* method appeared to be a useful technique for assessment of the ability of different lymphocyte subpopulations to respond to mitogenic activation.

Influence on the defence system

A limited number of studies is available concerning the effects of some quinolones on immune functions in man, rats and mice (Descotes, 1988; McDonald and Pruul, 1990). These comprehensive reviews report studies which mainly relate to *in vitro* tests regarding mitogen induced T-lymphocyte proliferation and several phagocyte functions, including antigen uptake, superoxide production, intracellular killing and chemotaxis. The results ranged from suppression to no effect or stimulation of these functions depending on the type of drug, the species and the experimental target parameter used. However, overall it was concluded that although some quinolones modify certain *in vitro* immune functions, these changes are relatively small and do not have *in vivo* correlates. In other words there is no evidence that any of the quinolones tested may lead to clinically relevant immunomodulation in mammals. In fish, research on immmunomodulating effects of quinolones is even more scarce than in mammals, while such information with regard to flumequine was completely lacking at the start of the present study.

Therefore and in view of the common use of flumequine in Dutch eel culture, it was decided to study the influence of this drug on the *in vivo* proliferative capacity of peripheral blood lymphoid cells in eels (chapter 5). The LPS response appeared to be enhanced by the drug, whereas a slight, but not significant, decrease was observed in the response to Con A after flumequine treatment. Moreover, flumequine proved to posses mitogenic properties by itself since the proportion of proliferated cells was significantly increased in comparison with untreated animals. Taking into account that flow cytometric analyses showed a significant reduction of the percentage of surface Ig positive (sIg^+) lymphoid cells in flumequine treated animals, the results point to a potentiation of sIg^- lymphoid cells by this drug. The latter agrees with the observation that flumequine did not significantly affect the production of circulatory antibodies against the intramuscularly injected antigen DNP-KLH (dinitrophenyl-keyholelimpet haemocyanin) in eel, as measured by ELISA (Van der Heijden *et al.*, 1994a).

Recently, Boon *et al.* (1996) reported a significant stimulation of the metabolic

activity of peripheral blood phagocytes in eel, after flumequine injection and subsequent *ex vivo* measurement of nitro blue tetrazolium dye reduction by those cells. Upon stimulation, mammalian phagocytes show an enhanced production of cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF), which directly and indirectly activate lymphocytes and themselves (Roitt *et al.*, 1993). IL-1 has been shown to potentiate both the specific and non-specific defence of mice due to its ability to activate T-lymphocytes (Cheers *et al.*, 1990). There are several indications that quinolones elicit production of certain cytokines, principally IL-1 and TNF, in human and rodent phagocytes (McDonald and Pruul, 1990; Bailly *et al.*, 1991; Stünkel *et al.*, 1991). Unfortunately, comparable data for fish are not available. However, in view of the observed immunostimulatory effects, it seems plausible that similar processes may occur in eel as both IL-1-like and TNF-like molecules have been demonstrated in other teleost species (Secombes, 1991; Ahne, 1993; Verburg-van Kemenade *et al.*, 1995). Nevertheless, to confirm the supposed mechanism for the effect of flumequine on the defence system in eel, additional experiments are required in which the relevant cytokine levels as well as their influence on leucocytes have to be determined in the species concerned.

The immunomodulatory effects of flumequine, presented above, are in accordance with results of Siwicki *et al.* (1989), who report a metabolic activation of spleen neutrophils in rainbow trout immunized with *Yersinia ruckeri* O-antigen after injection of, the structurally related, oxolinic acid at doses from 0.1 to 10 $mg \cdot kg^{-1}$ BW. Unfortunately, they did not provide any data on the influence of the drug on the proliferative capacity of lymphoid cells. However, monitoring the specific immune response by means of a hemolytic plaque assay revealed that the numbers of antibody-producing cells were not significantly affected by the quinolone they used.

Disease resistance

The defence system is an exquisitely balanced, highly complex system involving an elaborate regulatory network and functionally interdependent immunocompetent cell populations. Alterations in this balance, being either depression or enhancement of a particular element, may be compensated or amplified by additional interactions or immunological feed back mechanisms (Van Loveren and Vos, 1991). As a consequence, the practical relevance of observed immunomodulation in terms of disease resistance can

not automatically be deduced from a statistically significant effect of a substance on defence mechanisms like e.g. phagocyte killing efficiency, antibody production or lymphocyte proliferation (chapter 5). In fact, any sound interpretation of such data should be based upon the observation that fixed criteria have been met in the course of exposure. But generally accepted critical values for immune alterations are not available yet. Therefore, challenge tests will have to be performed to evaluate the predictive significance of basic immunological changes towards disease resistance.

Over the last decade quite a number of studies has been dedicated to infections of European eel with the commonly occurring parasitic swimbladder nematode *Anguillicola crassus*. As a result of these studies, a gradually optimized , well functioning infection model of *A. crassus* in European eel became available (Haenen *et al.*, 1989; Boon *et al.*, 1990a; Boon *et al.*, 1990b; De Charleroy *et al.*, 1990). In chapter 6 a challenge study using experimental infection of flumequine- or oxytetracycline-treated European eel with this parasite, is described. The percent recovery of parasites in the swimbladder, 8 weeks after inoculation, was used to quantify some association between drug treatment and host resistance. The experiment ultimately revealed a parasite recovery in flumequine-treated eels which was considerably lower than in non-medicated controls and significantly lower than in eels treated with oxytetracycline. These results indirectly affirm the enhancement (chapter 5) and impairment (Rijkers, 1980; Grondel, 1986) of the fish defence system by the respective drugs, while they simultaneously demonstrate the relevance of this influence for the protection against an invading pathogen.

The observed differences in protection against *A. crassus* indicate the suitability of this challenge model for assessment of the impact of immune alterations in European eel. Unfortunately, the exact mechanisms involved in resistance against *A. crassus* are not yet fully elucidated (Haenen, 1995). So, the model must actually be regarded as a 'black box'; the infection dose is known and the resulting nematode burden can easily be determined, but everything happening in between remains to be investigated. This flaw was recognized and might have been largely avoided by using a bacterial challenge model, since the existing knowledge on immunity of fish against bacterial infections is generally on a much higher level. However, living bacteria could not be used in the present case, because a possible toxic effect of the antibacterial drugs on the microbes would interfere with the results. On the other hand, application of killed bacteria or bacterial fractions, as an obvious alternative, was considered to be too remote from a natural challenge to yield meaningful information on the degree to which fish can really

resist infections.

A. crassus infestations in European eels were reported to be accompanied by a shift in the peripheral white blood cell picture of the host (Boon *et al.*, 1990b; Höglund *et al.*, 1992) and by production of specific antibodies (Buchmann *et al.*, 1991; Höglund and Pilström, 1994). Consequently, during the challenge study with this parasite (chapter 6), the above parameters were chosen to monitor the course of the host reaction, as well as the influence of the drugs upon this process. The leucocyte counts (i.e. granulocytes, total lymphoid cells and B-lymphocytes) were indeed clearly raised by the infection, which is indicative of a humoral and cellular response to the parasite. However, despite significant differences, changes in the numbers of leucocytes were difficult to interpret as far as effects of the drugs are concerned. Furthermore, anti-*A. crassus* antibodies could not be demonstrated, irrespective of the antibacterial treatment. This absence of a specific humoral response against *A. crassus* after a single experimental (re)infection was confirmed by Haenen *et al.* (1995). Recent results indicate that multiple reinfections (at least twice weekly for 4 weeks; K. Knopf, pers. comm.) are required to elicit demonstrable specific antibody titres under experimental conditions.

The fact that specific antibodies were not found implies that the drug-induced difference in resistance against *A. crassus* has to be attributed to an effect on other forms of defence. This supports an actual causative connection between the improved protection in eels treated with flumequine and the apparent immunostimulating properties of this drug, which relate to cellular (chapter 5) and non-specific (Boon *et al.*, 1996) defence mechanisms. Accordingly, it is concluded that flumequine may augment resistance against infections in eel through a beneficial effect on the defence system.

Use of antibacterial drugs in eel culture

Unlike in Japanese pond culture of eels, bacterial disease problems in European eel reared under controlled conditions in closed recirculation systems are mainly attributed to facultative fish pathogenic bacteria, whose pathogenicity depends on the quality of the environment and the associated fish health condition. This means that at least the majority of the bacterial diseases of European eel may be prevented. As indicated previously, good farm management, including water quality control, strict hygiene and regular health monitoring (providing the possibility of early intervention), is crucial in

this situation. Prophylactic administration of antibacterial drugs is usually neither feasible nor sensible because the problems are predominantly caused by ubiquitous bacteria, which will quickly recover once the drug concentration drops below their minimum inhibitory concentration. Next to a small chance for success, this procedure additionally carries the disadvantages known to be associated with the use of drugs. Notably the risk of development of drug resistant bacterial pathogens may have serious consequences if disease really strikes.

In case of an (acute) outbreak of bacterial disease, treatment with antibacterial drugs may be necessary to lower mortalities and to allow partial recovery of the fish. However, such treatment is not likely to be effective without supporting environmental optimization, while the use of immunosuppressing drugs may further weaken the immunocompromised animals. Moreover, to achieve satisfying results, (confirmation of) drug selection should be based on drug sensitivity tests with the isolated pathogen and, of course, on the pharmacokinetic characteristics of the drugs. Unfortunately, knowledge about the latter is still very restricted for European eel and certainly requires further investigation so as to meet the chemotherapy needs of the eel culture industry and to ensure the safety of eel for human consumption.

Flumequine, one of the few drugs investigated in this respect, appeared to exhibit a number of beneficial qualities regarding the efficacy to be expected. The drug is rapidly absorbed from the intestinal tract, shows good tissue distribution and remains above the minimum inhibitory concentration of most important eel pathogenic bacteria for quite a long time (chapters 2, 3). This, in combination with a beneficial influence on the natural resistance (chapters 5, 6), may explain many effective treatments and hence the repeated selection of this drug. Conversely, the long persistence in eel plasma and tissues, as well as the fact that the drug is probably almost exclusively excreted in unchanged form (Boon *et al.*, 1991), constitute some major disadvantages of flumequine from the viewpoint of public health and environmental protection. Virtually impracticable withdrawal periods of about 5 to 6 months (according to data from Boon *et al.* (1991) and chapter 2, respectively), which are needed to safeguard the consumer against (detectable) residues, make the drug unfit for application in the final phases of eel culture.

Considering that flumequine proved to augment the eels' defence system (chapter 5) and their resistance against disease (chapter 6), the drug does seem suitable to treat bacterial diseases in early culture phases, at least in as far as there is no risk for residues

in consumable eels. However, if the drug is not adequately removed from the holding water after treatment, difficulties are still to be expected since residual antibacterial drugs, and especially the highly stable quinolones, can persist for a long time in recirculation systems (Lunestad, 1992; Schlotfeldt *et al.*, 1995). This may lead to repeated uptake of the drug, resistance among bacterial pathogens, and damage to the nitrifying bacteria in the bio-filter. Therefore, and also in order to keep up with tightening regulations on draining of contaminated effluent, precautionary measures, in the form of specially designed filters for drug removal (Marking *et al.*, 1990; Smith *et al.*, 1994), may be required.

Some other major drugs applied to control bacterial diseases in European eels are oxytetracycline and furazolidone. However, on the basis of the presently available knowledge, these drugs do not seem to be really suited for this purpose. Pharmacokinetic data of oxytetracycline in eels, originating from a residue study by Nouws *et al.* (1993), reveal that the drug is poorly eliminated ($t_{½\beta}$ in plasma of 215 h after intramuscular injection of 60 $mg \cdot kg^{-1}$ body weight at 22°C) and accumulates in bone tissue. Furthermore, suppression of the defence system, as repeatedly demonstrated for oxytetracycline (Grondel and van Muiswinkel, 1986; Descotes, 1988; chapter 6), should definitely be avoided in a situation of decreased resistance, which is supposed to be the root of bacterial disease in European eels kept under intensive conditions. Objections concerning the use of furazolidone, are not so much directed towards the mother compound, which appeared to be rather rapidly cleared from the blood of European eels ($t_{½\beta}$=5.5 h after peroral administration of 50 $mg \cdot kg^{-1}$ body weight at 23°C; Van der Heijden *et al.*, 1994b), but relate to a persistent protein bound metabolite with carcinogenic potential (Nakabeppu and Tatsumi, 1984; Vroomen *et al.*, 1986; Vroomen *et al.*, 1988). So, the possible hazards due to the use of this drug far outweigh any advantage. Accordingly, the interest in furazolidone and other nitrofuran derivatives for aquaculture use decreased and their use in food animals has now largely been abandoned in many countries, including The Netherlands. Application of these questionable drugs in eel culture, after all, may finally harm the public opinion about the production methods and the fish produced.

Despite the fact that intensive eel culture in north-western Europe exists for over 20 years, knowledge with regard to antibacterial treatment methods have significantly lagged behind the developments in this sector and might be considered as inadequate. Therefore, research to support the registration of antibacterial drugs is urgently needed

so as to gain legal disposal of a panel of appropriate drugs in the end. At present, over 20 different antibacterial drugs are approved by the Japanese government for use in their indigenous eel species (Okamoto, 1992). Unfortunately, any reports which actually verify the suitability of these drugs are either absent or not accessible. Nevertheless, some of these drugs might appear to be useful in the European situation as similarities in drug behaviour and toxicity between the two species are expected on account of their close anatomical and physiological resemblance.

Final considerations

In general, there is a worldwide lack of drugs, especially antibacterial drugs, for aquaculture use (Schnick, 1992). In most aquatic farming countries, relatively few (and sometimes even no) drugs are registered to combat bacterial diseases in food fish, despite the fact that these diseases constitute the largest single cause of economic losses in aquaculture (Meyer, 1991). The shortage will be mainly due to the large variety in existing regulations applied to aquaculture drugs from one country to another and to the generally high costs for the pharmaceutical industry to satisfy national registration requirements as against a comparatively small market. To overcome the actual impasse actions are needed to attract the indispensable interest of the pharmaceutical industry for the aquaculture sector, without having to make concessions towards the efficacy or safety of drugs. Uniformity in the range of permissible treatments, regulations on residue limits and necessary chemical-analytical methods among different countries could enlarge the markets for aquaculture drugs, which may result in an extended scope for tailoring these drugs to a specific application. At the same time the comparatively important trade in aquaculture animals and products could benefit by the arising possibilities to facilitate im- and export procedures. Therefore, and in view of the nature and extent of the problem, steps towards harmonisation of regulations by far-reaching cooperation of agencies concerned, on the level of the European Union (EU), or preferably even on a more comprehensive international scale, seems to be the most suitable approach. A process which should finally lead to central registration of drugs in the EU is currently developing, however, without concrete results for the aquaculture sector as yet.

All veterinary drugs, which are put forward for registration, are evaluated for quality and for their efficacy to control the target pathogens, but also for safety with regard to

the consumer, the environment and the animals for which they are intended, before official approval by the authorities. The impact of a drug on the target animal's health is usually examined by routine toxicological studies, but data with regard to possible modulating effects on the defence system are not necessarily required. This is regrettable, because on the one hand, the use of immunosuppressing drugs, which impair the natural resistance of an animal, may eventually end in a vicious circle of infectious disease and drug (ab)use. On the other hand, the combined action of a non-compromised defence system and an immunostimulating drug will increase the defensive potential and consequently support a rapid recovery of the diseased fish. Therefore, it is strongly recommended that antibacterial drugs are systematically examined for possible unintentional immunological effects and that these data are taken into account during future registration procedures.

Unfortunately, at present there is still no question of a structural approach concerning the research on immunomodulating effects of drugs. Results are usually difficult to compare as a consequence of differences in several test variables and/or a lack of standardization within test protocols. Notwithstanding the risk that standardization discourages creativity and restrains the development of new test approaches, a certain degree of standardization is certainly needed especially if the tests are to be used as regulatory tools.

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Summary

Impact of antibacterial drugs on the health of European eel

The introduction of intensive culture techniques in fish farming, was accompanied by an increased prevalence of infectious diseases as a result of a combination of a high infection pressure and decreased resistance of the animals. To reduce losses due to bacterial infections, fish are treated with antibacterial drugs. These drugs are generally of remarkable value for the control of bacterial infections. However, especially in case of excessive use, they may have a number of adverse effects. In this respect there is a growing concern about development of resistant bacteria, drug residues in food fish and environmental pollution, but also about a possible negative influence on the target animals' health (**chapter 1**).

During the last decades, several studies have shown that certain antibacterial drugs, including some drugs commonly applied in fish culture, exhibit side effects in the form of inhibition or stimulation of the immune system. Any antibacterial drug-induced immunosuppression, however, is greatly undesirable, because an optimal immune function is important for both the final elimination of the pathogen that gave rise to the therapy and the resistance against a possible new infection. Stimulation of the defence system, on the other hand, may be regarded as a beneficial effect, which fully fits within the purpose of an antibacterial treatment.

Positive as well as negative effects of drugs depend partly on the concentrations of the substance in the host following administration. Accordingly, in **chapter 2** a pharmacokinetic study is described, using flumequine, a second generation quinolone derivative which is commonly used in intensive aquaculture, in three important cultivated fish species in The Netherlands, namely common carp (*Cyprinus carpio* L., 1758), African catfish (*Clarias gariepinus* Burchell, 1822) and European eel (*Anguilla anguilla* L., 1758). The plasma disposition of the drug was compared between the respective species after a single peroral administration at a customary dose of 18 $mg \cdot kg^{-1}$ body weight. All species showed rapid absorption followed by biphasic plasma depletion. Apart from that, significant interspecies differences were observed for most of the determined pharmacokinetic parameters. Mean highest plasma flumequine concentrations were, in decreasing order, 6136 $ng \cdot ml^{-1}$ in carp, 4074 $ng \cdot ml^{-1}$ in eel and 1179 $ng \cdot ml^{-1}$ in catfish. Moreover, calculation of the mean distribution and elimination

half-lives revealed 3.4 and 104.3 h for carp, 7.3 and 59.5 h for catfish and 56.7 and 451.2 h for eel. So, to achieve equivalent drug efficacy and to minimize the probability of residues as a result of medication, dosage regimes and withdrawal periods should be adapted for each species.

Most striking was the extremely slow elimination of flumequine in plasma of eels. Hence, in **chapter 3** the tissue distribution and residue elimination of the drug was studied in more detail in this species after a single intramuscular injection. Concentrations of flumequine in muscle and liver were respectively similar and higher than in plasma during the investigated period (44 days). Elimination of the drug from the examined tissues was very slow and in the same order of magnitude as from the plasma. After an observation period of more than 6 weeks the mean drug concentration in plasma and different tissues was still above 85 $ng \cdot ml^{-1}$ or $\cdot g^{-1}$, which is higher than the minimum inhibitory concentration (MIC) reported for most important eel pathogenic bacteria. This may (partly) explain the use of flumequine in eel culture, but also emphasizes the aforementioned risk for residues of this drug in eels destined for consumption. In addition, the long drug persistence in plasma and tissues makes eel a suitable model to study a possible influence of flumequine on the natural resistance and the associated health condition.

Lack of appropriate test assays for *in vivo* or *ex vivo* determination of suitable immunological parameters necessitated preliminary development of some tools. To this end, monoclonal antibodies (mAbs) to European eel immunoglobulin (Ig) were produced, characterized and tested for applicability in a number of immuno(cyto)chemical assays (**chapter 4**). The selected mAbs, WEI 1 and WEI 2, were specifically reactive with Ig heavy and light polypeptide chain, respectively. WEI 1 was reactive with all or nearly all Ig molecules, B cells and plasma cells, whereas WEI 2 reacted with a subpopulation of those cells. Therefore, the former mAb was the most suitable reagent for flow cytometric determination of Ig-bearing (B) lymphocyte proportions. However, both mAbs appeared to be equally useful for detection of antigen-specific antibodies in plasma by means of an enzyme-linked immunosorbent assay (ELISA).

In **chapter 5** the influence of flumequine on the proliferative capacity of lymphoid cells in eels was studied. For this purpose a newly developed *in vivo* test was applied. Essentially, this test included peroral drug administration followed by successive intraperitoneal injections with a mitogen, being either concanavalin A (Con A) or

lipopolysaccharide (LPS), and the immunocytochemically demonstrable thymidine analogue 5-bromo-2'-deoxyuridine. Subsequent counting of proliferated cells in peripheral blood smears revealed that flumequine itself possesses mitogenic properties. Moreover, the drug appeared to enhance the proliferative response of lymphoid cells to LPS, whereas the Con A response was not significantly affected after flumequine treatment. Flow cytometric analysis of peripheral blood lymphoid cells showed a significant reduction of the proportion of surface immunoglobulin positive cells in the flumequine-treated animals. It was concluded that flumequine enhances proliferation of lymphoid cells (probably surface immunoglobulin negative cells) in eels.

The influence of flumequine on a basic immunological mechanism, as described above, does not allow a sound judgement of the functioning of the entire defence system with regard to resistance to infections. Therefore, in **chapter 6** the effect of flumequine and also oxytetracycline, an antibacterial drug with known immunosuppressive properties, was finally investigated by means of a challenge test. The eels were experimentally infected with *Anguillicola crassus* (Kuwahara, Niimi et Itagaki, 1974), a commonly occurring eel parasitic nematode residing in the swimbladder. Eight weeks after oral administration of infective larvae, the mean recovery of the parasites in flumequine-treated eels was lower than in the non-medicated control animals and significantly lower than in oxytetracycline-treated eels. Mean numbers of peripheral blood granulocytes and B-lymphocytes as well as the total amount of circulating lymphoid cells showed an increase as a result of the infection, while drug treatment significantly affected the quantities of the lymphoid cells. The difference in protection against the parasite after flumequine or oxytetracycline administration, pointed to a modulation of the fish resistance as a result of the drug treatments. The fact that specific antibodies to the parasite were not found favoured an effect on the cellular rather than on the humoral response, which is in accordance with previous suggestions.

The immunostimulating effect and the pharmacokinetic characteristics of flumequine which appeared from the present study, in combination with the MIC values reported for a number of important pathogens, indicate that flumequine can be an effective drug to control bacterial infections in eel. However, the slow elimination and the long withdrawal times which are consequently needed, make the drug unsuitable for routine application in eel culture (**chapter 7**).

Samenvatting

Invloed van antibacteriële medicijnen op de gezondheid van Europese aal

De invoering van intensieve houderijmethoden in de visteelt, ging gepaard met een verhoogde prevalentie van infectieziekten ten gevolge van een combinatie van een hoge infectiedruk en een verminderde weerstand van de dieren. Om verliezen ten gevolge van bacteriële infecties te beperken worden antibacteriële medicijnen aan de vissen toegediend. Het is algemeen bekend dat deze middelen buitengewoon belangrijk zijn voor de beheersing van genoemde infecties. Vooral bij overdadig gebruik kunnen deze echter een aantal nadelige effecten hebben. In dit verband is er een toenemende bezorgdheid over het ontstaan van resistente bacteriën, geneesmiddelresiduen in consumptievis en de verontreiniging van het milieu, maar ook over een mogelijk negatieve invloed op de gezondheid van de behandelde vissen (**hoofdstuk 1**).

Gedurende de afgelopen decennia werd meerdere malen aangetoond dat bepaalde, ook in de visteelt algemeen toegepaste, antibacteriële geneesmiddelen bijwerkingen vertonen in de vorm van remming of stimulatie van het immuunsysteem. Iedere immunosuppressie als gevolg van antibacteriële medicatie is echter hoogst ongewenst, omdat een efficiënte werking van het afweersysteem zowel van belang is voor de uiteindelijke eliminatie van het pathogeen waarvoor de therapie werd ingesteld, als voor de weerstand tegen een eventuele nieuwe infectie. Stimulatie van de afweer kan daarentegen worden beschouwd als een gunstig effect, dat volkomen past bij het doel van een antibacteriële therapie.

Zowel positieve als negatieve effecten van geneesmiddelen zullen gedeeltelijk afhankelijk zijn van het concentratieverloop van het middel in het dier. Dienovereenkomstig werd in **hoofdstuk 2** begonnen met een farmacokinetisch experiment met gebruikmaking van flumequine, een tweede generatie quinolone derivaat dat algemeen wordt toegepast in de intensieve aquacultuur, en drie voor de Nederlandse situatie belangrijke vissoorten, te weten gewone karper (*Cyprinus carpio* L., 1758), Afrikaanse meerval (*Clarias gariepinus* Burchell, 1822) en Europese aal (*Anguilla anguilla* L.,1758). De afname van de concentratie van het geneesmiddel in plasma werd vergeleken bij de respectieve soorten na eenmalige perorale toediening van een gebruikelijke dosis van 18 $mg \cdot kg^{-1}$ lichaamsgewicht. Alle drie de soorten vertoonden een snelle absorptie gevolg door een bifasische afname van de concentratie in het

plasma. Verder werden er significante verschillen waargenomen tussen de soorten met betrekking tot het merendeel van de bepaalde farmacokinetische parameters. De hoogste gemiddelde flumequine-spiegels in plasma waren, in afnemende volgorde, 6136 $ng \cdot ml^{-1}$ in karper, 4074 $ng \cdot ml^{-1}$ in aal en 1179 $ng \cdot ml^{-1}$ in meerval. Berekening van de gemiddelde distributie- en eliminatie-halfwaardetijden leverde voorts 3.4 en 104.3 h voor de karper, 7.3 en 59.5 h voor de meerval en 56.7 en 451.2 h voor de aal. Dit betekent dat voor een gelijkwaardige werkzaamheid en ter voorkoming van residuen in consumptievis, dosering en wachttijd dienen te worden aan gepast per soort.

Zeer opvallend was de uitermate trage eliminatie van flumequine in plasma van de aal. Derhalve werd in **hoofdstuk 3** uitvoeriger gekeken naar de verdeling over de verschillende weefsels en de eliminatie van residuen van het middel in deze vissoort na een eenmalige intramusculaire injectie. De concentraties van flumequine in spier en lever waren respectievelijk overeenkomstig en hoger dan de in plasma gemeten concentraties gedurende de bestudeerde periode (44 dagen). De eliminatie van het middel uit de onderzochte weefsels was zeer langzaam en lag in dezelfde orde van grootte als de eliminatie uit plasma. Na ruim 6 weken was de flumequine-concentratie in plasma en de verschillende weefsels nog steeds hoger dan 85 $ng \cdot ml^{-1}$ of $\cdot g^{-1}$, hetgeen hoog genoeg is voor remming van de meest belangrijke, voor aal pathogene bacteriën. Dit zou (deels) het gebruik van flumequine in de aalmesterij kunnen verklaren, maar benadrukt tevens het eerder genoemde gevaar voor residuen van dit geneesmiddel in voor de consumptie bestemde aal. De lange verblijfsduur van het farmacon in plasma en de verschillende weefsels maakt aal bovendien tot een geschikt model voor de bestudering van een eventuele invloed van flumequine op de natuurlijke weerstand en de hieraan gerelateerde gezondheidsstatus van de vis.

Gebrek aan passende testen voor *in vivo* of *ex vivo* bepaling van geschikte immunologische parameters maakte echter een voorafgaande ontwikkeling van het benodigde 'gereedschap' noodzakelijk. Hiertoe werden monoklonale antilichamen (mAbs) tegen immunoglobuline (Ig) van de Europese aal gemaakt. Vervolgens werd het reactiepatroon van de mAbs gekarakteriseerd en werd hun toepasbaarheid in een aantal immuno(cyto)chemische testen nagegaan (**Hoofdstuk 4**). De geselecteerde mAbs, WEI 1 en WEI 2, vertoonden een specifieke reactie met respectievelijk de zware en lichte polypeptideketen van het Ig. WEI 1 reageerde met alle of nagenoeg alle Ig-moleculen, B cellen en plasma cellen, terwijl WEI 2 met een subpopulatie van deze cellen reageerde. WEI 1 was daarom het meest geschikt voor flow-cytometrische bepaling van

het percentage Ig-dragende (B) lymfocyten. Beide mAbs bleken echter in gelijke mate geschikt te zijn voor de detectie van antigeen-specifieke antilichamen in plasma met behulp van een 'enzyme-linked immunosorbent assay' (ELISA).

In **hoofdstuk 5** werd onderzocht in hoeverre flumequine invloed heeft op het proliferatievermogen van lymfoïde cellen in aal. Hiervoor werd gebruik gemaakt van een nieuwe *in vivo* test. Deze test behelsde in wezen perorale toediening van het geneesmiddel, gevolgd door intraperitoneale injecties van achtereenvolgens een mitogeen, hetzij concanavaline A (Con A) of lipopolysaccharide (LPS), en het immunocytochemisch aantoonbare thymidine analoog 5-bromo-2'-deoxyuridine. Door vervolgens het aantal geprolifereerde cellen in bloeduitstrijkjes te tellen bleek dat flumequine op zich mitogene eigenschappen bezit. Verder bleek het geneesmiddel een verhoogde respons op LPS te bewerkstelligen, terwijl geen significant effect gevonden werd op de proliferatie ten gevolge van Con A. Flow-cytometrische analyse van lymfoïde cellen in perifeer bloed toonde aan dat het relatieve aantal lymfocyten met membraangebonden Ig significant verlaagd was in de met flumequine behandelde dieren. Er werd zodoende geconcludeerd dat flumequine de proliferatie van lymfoïde cellen (waarschijnlijk degenen zonder membraangebonden Ig) in aal bevordert.

De invloed van flumequine op een elementair immunologisch proces, zoals hierboven beschreven werd, geeft echter geen volledig inzicht in het functioneren van het afweersysteem als geheel met betrekking tot de weerstand tegen infecties. In **hoofdstuk 6** werd daarom tenslotte het effect van flumequine alsmede oxytetracycline, een antibioticum met immunosuppressieve eigenschappen, onderzocht met behulp van een 'challenge experiment'. Aal werd hiervoor oraal geïnfecteerd met *Anguillicola crassus* (Kuwahara, Niimi et Itagaki, 1974), een algemeen voorkomende parasitaire nematode van de zwemblaas in aal. Acht weken na toediening van de infectieuze larven was het gemiddelde, in de zwemblaas aangetroffen aantal parasieten in aal behandeld met flumequine lager dan in de niet-gemedicineerde controlevissen en significant lager dan in de met oxytetracycline behandelde aal. De gemiddelde aantallen granulocyten en B-lymfocyten in perifeer bloed, evenals het totale aantal circulerende lymfoïde cellen, waren verhoogd als gevolg van de infectie, terwijl de geneesmiddelbehandeling een significant effect bleek te hebben op de hoeveelheden lymfoïde cellen. Het verschil in bescherming tegen de parasiet na toediening van flumequine dan wel oxytetracycline wees op een modulatie van de weerstand ten gevolge van deze behandelingen. Het feit dat geen specifieke antilichamen tegen de parasiet werden gevonden, gaf aan dat het

waargenomen effect eerder moet worden toegeschreven aan een invloed op de cellulaire dan op de humorale respons, hetgeen overeenstemt met suggesties hieromtrent in het voorgaande hoofdstuk.

Het immunostimulerende effect en de farmacokinetische karakteristieken van flumequine die uit het onderhavige onderzoek naar voren kwamen, in combinatie met de in de literatuur vermelde gegevens met betrekking tot de gevoeligheid van een aantal belangrijke pathogenen voor flumequine, geven aan dat het een effectief middel kan zijn tegen bacteriële infecties in aal. Gezien de trage eliminatie en de hierdoor vereiste lange wachttijden is het middel echter niet geschikt voor routinematig gebruik in de aalmesterij (**hoofdstuk 7**).

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Tom van der Heijden,
Wageningen, februari 1996.

Curriculum vitae

Tom van der Heijden werd op 19 april 1963 geboren in Wittem (Zuid-Limburg). Na de basisschool te hebben doorlopen op het jongenspensionaat te Kerkrade werd in 1982 het VWO-diploma behaald aan de scholengemeenschap 'Sophianum' te Gulpen. Vervolgens werd in 1983 begonnen met een studie Biologie aan de toenmalige Landbouwhogeschool te Wageningen. Na een brede biologische basis gelegd te hebben, met een nadruk op zoölogische vakken, werden in de doctoraalfase leeronderzoeken verricht op het gebied van Visgezondheid en Immunologie, uitgevoerd bij respectievelijk de vakgroep Visteelt en Visserij en de vakgroep Experimentele Diermorfologie en Celbiologie. Op 25 januari 1991 werd het ingenieursdiploma in de Biologie in ontvangst genomen aan de Landbouwuniversiteit. In datzelfde jaar nog kon, na in dienst te zijn getreden als assistent in opleiding (AIO) bij de vakgroep Visteelt en Visserij, worden gestart met een promotieonderzoek waarvan de resultaten in het thans voor u liggende proefschrift beschreven zijn. In het kader van AIO-onderwijs werd onder andere de cursus Pathobiologie, gegeven aan de Faculteit Diergeneeskunde van de Rijksuniversiteit Utrecht, met goed gevolg afgesloten. Als onderwijstaak werd geassisteerd bij het practicum Visgezondheid en werd een aantal studenten begeleid tijdens hun doctoraal onderzoek.

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