

LABORATORY OF ICHTHYLOGY, FISH DISEASES & AQUACULTURE

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Limitations and shortfalls in parasitic control in Mediterranean aquaculture: future needs

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Brief overview of main parasitic diseases

- **Protozoa:** *Amyloodinium*, *Trichosina*, *Cryptocaryon* spp
- **Monogenea:**
 - *S. aurata*: *Funestrinia*, *Microcotyle* spp.
 - *D. labrax*: *Diplectanum* spp.
 - *D. puntazzo*: *Atrispinum salpae**, *A. seminalis*, *Sparicotyle chrisophrii*, *Polylabris tubicirrus*
 - Problem: Enlargement of host range under aquaculture conditions!
- **Isopoda:** *Ceratothoa oestroides*
- **Copepoda:** *Lernathropus kroyeri*, *Caligus minimus*, *C. mugilis* in *D. labrax* (lagoons)
- **Myxosporea:** *Enteromyxum leei*, *Polysporoplasma sparis*, *Ceratomyxa* spp., *Myxobolus* sp (*D. puntazzo*)

Emergence of “new” parasites as pathogens

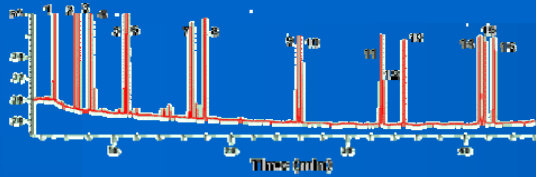
- *Amoeba*-like organisms
- *Cryptosporidium molnari*
- *Myxobolus* sp.
- *Monogenea*: *A. seminalis*, *Sparicotyle chrisophrii*, *Polylabris tubicirrus*
- *Caligus* spp.



Parasites of wild fish

- Relationships between wild and farmed stocks and pathogens are complex and poorly researched
- Ongoing research on wild fish neighboring cages in Greece :
 - Species examined:
 - (*Pagelus acarne* L. - Sparidae)
 - (*Pagelus erythrinus* L. - Sparidae)
 - (*Boops boops* L. - Sparidae)
 - (*Diplodus annularis* L- Sparidae)
 - (*Serranus* sp.- Serranidae)
 - (*Trigla* sp. - Triglidae)
 - (*Uranoscopus scaber* L. -Uranoscopidae)
 - (*Merluccius merluccius* -Gadidae)
 - (*Mullus barbatus* L.-Mullidae)
 - ***Mugil cephalus* (Mugilidae)**
- Few positive cases for ***E. leei* and trematodes esp. Mugilids**
- *further confirmed by the preliminary results of a wider investigation through an EEC project concerning 4 different Mediterranean countries by traditional methods and novel PCR test (44/2019 fish examined +ve for E. leei)*
- ***Preliminary results of wild fish parallell those of reared sea bream in near by sites i.e. : no clinical signs, no infection obvious by traditional tests but higher prevalence in PCR testing .***





Current treatments and preventive methods

- The main parasitic diseases of cultured Mediterranean fish causing problems (mortalities, delay in growth rates and / or loss of fecundity) and requiring treatments are the **Myxosporeans** (*E. leei*, *P. sparis*), **Isopods and Copepods**. In some areas there are also heavy mortalities due to Monogenea infections.
- **TREATMENT OF MONOGENEA**
 - . By formalin
 - *Limitations: concerns for safety of personnel and fish (anoxia), paraformaldehyde formation below 5 °C, toxicity at high T>21 °C, sensitive species (hybrid striped bass)*

TREATMENT OF ISOPODA/ CALIGIDAE

No licensed drugs exist for use in Mediterranean species.

Drugs used in salmonid industry have problems :

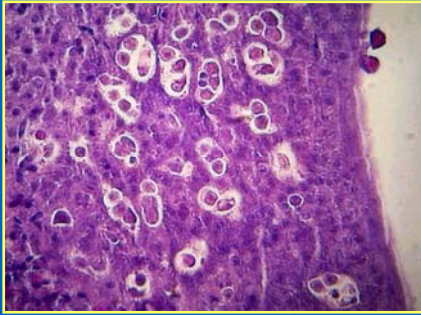
- Organophosphates: very toxic especially the adjuvant
- Diflubenzuron, Teflubenzuron: effective, per os, some environmental effects but a good candidate
- Deltamethrin: Alphamax, Excis: effective but very expensive, bath treatments
- Ivermectin: very effective, per os and baths, but environmental problems
- Avemerctin (Slice): per os, not widely tested in Mediterranean fish, but, preliminary results are disappointing

Preventative measures comprise:

- Good quality sites selection (adequate water flow, good water quality)
- Year class separation combined with fallowing of sites
- Proper management and clean nets
- Addition of light at the corners of the cages

Limitations: not effective treatment most of the times.





Treatment of Myxosporea:

- So far only **Fumagillin** and its analogs (abroad) was used, but has serious side effects (immunosuppression)
- *Limitations:*
- Fumagillin is not effective in many pathogenic species (*M. leei*).
- High doses of the drug are lethal, causing haemorrhage and congestion of the liver and tubular degeneration of the kidney whereas low doses produce reversible changes in haematopoietic tissue only in the kidneys (aplastic anaemia).
- The use of Fumagillin involves prolonged treatment periods and is consequently expensive
- No information on intermediate/alternative hosts exists in most marine species
- No rapid method for early detection commercially exists as yet.

RECENT AND ONGOING RESEARCH

1. Research on novel treatments:

– *Research on Monogenea*

- There is limited work done in recent years concerning the treatment of Monogenea in Mediterranean cultured fish. Preliminary experimental treatments with **ivermectin** at levels used for sea lice in salmon in sea bass has been promising.

– *Research on Isopoda/Copepoda*

- Ivermectin
- Deltamethrin (Alphamax)
- Diflubenzuron



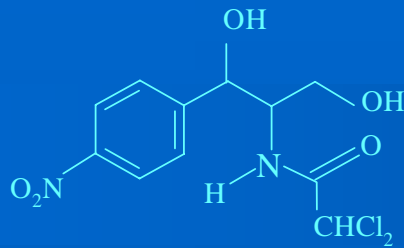
Results: Ivermectin

Toxicity of ivermectin in sea bass after administration via different routes.

Route of Administration	Fish Weight (g)	Temperature (oC)	No. Fish/ Concentration	LD50 ¹ (mg/kg)	95% Confidence Interval	
Intubation	3	20	100	0.839	0.628	1.121
Intubation	3	11	60	0.335	0.299	0.375
Intubation	35	11	25	0.523	0.340	0.805
Injection	3	20	100	1.023	0.739	1.415
Injection	3	11	100	0.106	0.083	0.136
Injection	35	11	25	0.361	0.284	0.458

¹LD50 values based on 40 day cumulative mortality.

- No signs of toxicity were observed
- The compound was found to be significantly more toxic to fish reared at 11°C than 20°C
- The therapeutic effect of the drug was very satisfactory in copepod (*Lernathropus kroyeri*) and *Monogenea* infections of sea bass at doses similar to those used in salmon



Deltamethrin experiments

- **1st:** 10, 1, 0,1, 0,01, 3, 0,3 mg/L X 0,5 ώρα
18 °C, pH =7,74, O₂ =5,2mg/L
Check at : 0,5, 2, 12, 24, 48 hr
- **2nd:** 0,05, 0,1, 0,15 ug/L X 0,5 ώρα
23 °C, pH =7,74, O₂ =5,2mg/L
Check at : 0,5, 2, 12, 24, 48 hr
- **3rd:** 10, 5, 3, ug/L & 0,05 mg/L X 0,5 ώρα
23 °C, pH =7,74, O₂ =5,2mg/L
Check at : 0,5, 2, 12, 24, 48 hr
- **Best results achieved at the dose of 10g/L where prevalence reduced from 100% to 0\$ over 24hr in both large and small fish.**

Di-flubenzuron results

Effect of treatment (% survival) with di-flubenzuron on the survival of *C. oestroides* in sea bass. (3mg/Kg/day X14 days), N=20fish.

Day	Control Small fish)	Control Larger fish)	Treated small fish	Treated larger fish
0	18/21	15/20	18/20	17/20
14	0/21	0/20	0/20	0/20
30	0/21	0/20	0/20	0/20

•Medicated pellets containing 3mg/Kg BW di-flubenzuron effectively cleared pre adult and adult stages of the isopod parasite over a period of 14 days.

•No adverse effects were recorded in treated sea bass during the trials and no re-infection occurred after 15 days of the end of the treatment

RECENT AND ONGOING RESEARCH

2. Research on novel treatments:

– *Research on Myxosporea*

1. Through extensive nationally funded research in years 1997-2002 concerning the immunology and treatment of different myxosporeans of cultured *Sparus aurata*, *D. puntazzo* and sea bass and *D. sargus* . Results are now published.
2. Through an EEC project: ‘Disagnosis, epidemiology, and control of enteric myxosporidiosis of commercial Mediterranean fish’ Sept. 2002-2006

Compounds used in experimental treatments for Myxosporea in

Mediterranean fish (*S. aurata* & *D. puntazzo*)

Compound	Dose	Scheme	Comments
Fumidil-1 (F)	2 -up to 25mg/kg	3 and 6 weeks	2 land based experiments * & 1 exp. field trial
Toltrazuril-2	600ml/T biomass	2 days on, 3 days off, 2 days on, repeat after 15 days	2 land based experiments
Toltrazuril-2	600ml/T biomass	2 days on, repeat after 15 days	2 land based experiments
Amprolium	190g/T biomass	30days	2 land based experiments
EsB-3	200g/T biomass	3days on, repeat after 15 days	2 land based experiments
Ampr+EsB-3	100+100g/T biomass	30 days	2 land based experiments
Ampr+Salilomycin12% (SA)	100+70g/T biomass	30days	22 land based experiments * & 1 exp. field trial
Oregano oils (Ecodiar) (O)	8-12ml/5Kg biomass	30 days	2 land based experiments * & 1 exp. field trial

Chemotherapy conclusions:

Treatments of Myxobolus sp in D. puntazzo and P. sparis in S. aurata

- In all three experiments and in both species, the treatment with **Amprolium and Sanilomycin 12% (SA)** was the most effective in terms of both therapy and toxicity to fish
- SA exhibited a significant reduction in **prevalence rate and intensity in both juvenile and adult fish**
- **No histopathological evidence for toxic side effects** was observed
- Oregano oils (O) also effective (50% reduction prevalence) but **ineffective in established infections of adult fish.**
- Fumagillin less effective than SA and O.
- There was a distinct reduction in the number of MMC in fumagillin treated fish. In contrast, in fish treated with SA, the inflammatory cells appeared only as limited areas and MMC remained stable, similar to normal fish, suggesting that MMC can be used as an indicator of drug toxicity

Immunology conclusions:

Treatments of *Myxobolus* sp in *D. puntazzo* and *P. sparis* in *S. aurata*

- *S. aurata* and *D. puntazzo* naturally infected with these myxosporeans exhibit **a state immunosuppression** (low lysozyme (L) secretion, phagocytic activity (PA), & lymphoproliferative capacity (Lphc))
- Treatment with SA was closely **correlated with an activation of phagocytic ability and capacity** as well as TNF-, IL-1-like, high NO-, and lysozyme secretion in a time dependent manner demonstrating an activation of the non specific immune system.
- Fumagillin and Oregano oils **failed to promote phagocytes functions** which coincides with the moderate effect of these drugs in controlling the infections.

RECENT AND ONGOING RESEARCH

3. Research on other aspects of *E. leei* (EEC project):

– *Aims:*

1. To provide and validate a reliable **PCR-based test** the use of oligonucleotide probes –based in variable regions of the *Myxidium leei* 18S rRNA gene
 - The use of this test will allow:
 - - *to know the epidemiological status in the different countries, coastal areas and farms and to evaluate the risk of infection.*
 - -*to control the introduction and dispersion of the disease in national and international fish transactions.*
2. Study of the mechanisms involved in **transmission, pathogenesis and infective process in sparid fish**, in the course of experimental infections
3. Study of relevant aspects of the **innate and adaptive immune responses** to the parasite, and evaluation of **immunomodulation strategies** for the prophylaxis of the infection.

E. leei in *D. puntazzo* and *S. aurata* epidemiology and experimental infections:

- **Positive samples of invertebrates** have been detected (Annelida, Anthozoa and amphipods) by PCR.
- Direct transmission through **co- habitation** of *E. leei* from donor naïve *D. puntazzo* to experimental- uninfected - fish (*D. puntazzo*) and *P. erythrinus* has been successful. **Oral intubation** less successful. Scarce initial stages have been identified . Infected fish become immunosuppressed. {This is in accordance with results of *S. aurata* exp. Infections}. PhD Thesis.
- Direct transmission through co- habitation of *E. leei* from donor naïve *D. puntazzo* to experimental **previously infected (survivors)** and **treated with SA fish** (*D. puntazzo*) has been unsuccessful. PhD Thesis.
- Treatments with **SA and oregano essential oils** resulted in reduction of prevalence up to **60% and 40%** respectively. PhD Thesis.
- Spanish sea bream and off springs more sensitive to innate (Greek) stock. PhD Thesis.

RECENT AND ONGOING RESEARCH

Research on immunity /vaccine development of parasites:

- **Sparidae:** some information on the immune system of sea bream exists but, mainly deals with non-specific mechanisms or responses against bacteria. No info on the response to myxosporeans or other parasites is available except for some **external protozoa** (*Amyloodinium*) and **trematodes** (*Microcotyle* spp)
- Preliminary data on ***D. puntazzo*** innate immune response is now generated and published through national projects. More info will be available through the EEC project.
- ***E. leei*:** The importance of immune responses at tissue level is important especially in the case of intestinal infections. Information on mucosal immunity in fish is scarcer, but the immunocompetence of its intestine has been demonstrated, involving GALT and other cellular populations. However, their role in myxosporoses or even other fish parasitoses is unknown.
- The development of an ELISA for the detection of specific antibodies is a useful tool and is only pending of some improvements.

RECENT AND ONGOING RESEARCH

4. Research on immunity /vaccine development of parasites:

Immunomodulation

- Most of the information deals with salmonid fish, but immunostimulation has also been assayed in other fish including gilthead sea bream (glucans vitamins, chitin but the best results with levamisole)
- Levamisole has been used in fish for treatments of infections and as immunostimulant or adjuvant. Hitherto, the use of immunomodulators has not been assayed to control any myxosporidiosis.

Vaccination

- Vaccination offers the prospects of a consumer-friendly and environmentally friendly effective control method for parasitic diseases
- Parasitic vaccines are still rare, but there are encouraging signs that their number will increase in the next decade (sea lice in salmon).

5. Research on immunity /nutrition of parasites:

‘EFFECT OF VARYING LEVELS OF VITAMINS C AND E ON GROWTH FEED UTILIZATION AND IMMUNE SYSTEM OF THE SHEEPHEAD BREEM (*Puntazzo puntazzo*)’. National grant.

D. puntazzo of about 3g were fed three experimental diets containing different vitamin C and E levels for seven months:

Diet A contained no additional vitamin C and 250 ppm of vitamin E, Diet B, 150 ppm vitamin C and 250 ppm of vitamin E and Diet C 300 ppm vitamin C and 250 ppm of vitamin E.

- growth and feed utilization were measured and leucocytes obtained from each group were analyzed for macrophage activity and their capacity to produce NO⁻, lysozyme, cytotoxic cytokines after in vitro stimulation.
- Specific growth rate and feed efficiencies did not show any significant differences among the formulations.
- According to the results fish exhibited higher macrophagic index, when were fed with diets B and C compared to fish fed with diet A. These results were in compliance with the levels of inducible NO⁻, lysozyme, and tumor necrosis factors in the supernatants of the above cultures
- Finally, the serum levels of NO⁻, lysozyme, and tumor necrosis factors were related to the myxosporean and trematode parasitic load.

The results suggest that for *S. aurata* diets with high levels of Vitamin C (300ppm) are necessary to significantly improve the non specific defense mechanism of this species under culture conditions.

FUTURE NEEDS & CONCLUSIONS

Research on:

- alternative treatments and management interactions (esp. sea lice)
- wild fish epidemiology, interactions (Myxosporeans, Isopoda)
- basic research on taxonomy, identification and life cycles of marine parasites
- early & rapid diagnostic methods for other myxosporeans and protozoa
- effective and environmentally friendly antifouling systems

Correct feeding and correlation with parasitic disease:

(soybean problem: FEEDING SOYBEAN PROTEIN!!!- *Myxobolus* (japan))

Management measures (quarantine, training, strategic therapies, veterinary presence and monitoring of diseases)

Regulation (importation, international trade, HACCP, notifiable diseases)

Minimize importation of various fish and different fish species, international trade of live fishes and shellfishes had introduced parasites hitherto unknown to several countries (case of Japan).

Technical improvement in seed production, disease resistant stock.



FUTURE NEEDS & CONCLUSIONS

- **Antiparasitic drugs** will remain important for a long time, although resistance development could limit their use . Need for strategic treatment in most parasites. Need for veterinary supervision & test trials!!
- **Many vaccines** might find their greatest and most immediate application in **integrated control strategies**
- **The combination of the correct treatments with other management and disease prevention strategies** (vaccinations, water quality improvement, production of tolerant fish, alternative treatments) and **further regulation** will help ensuring the successful development of the sector in the future.

questions ?

Thank you
very much
for your
attention !

