The fish parasite *Ichthyophthirius multifiliis* – Host immunology, vaccines and novel treatments

A review

By Louise von Gersdorff Jørgensen

Department of Veterinary and Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen
DK-1870 Frederiksberg C
Denmark

Abstract

Ichthyophthirius multifiliis, the causative agent of white spot disease (Ichthyophthiriasis) is a major burden for fish farmers and aquarists globally. The parasite infects the skin and the gills of freshwater fish, which may acquire a protective adaptive immune response against this disease, making vaccine strategies feasible. However, there is no prophylactic treatment available and repetitive treatments with auxiliary substances are needed to control the infection. Historically, a variety of drugs and chemicals have been used to combat the disease but due to changing regulations and recognition of carcinogenic and environmentally damaging effects the most efficient compounds are prohibited. A continuous search for novel substances, which are highly effective against the parasites and harmless for the fish is ongoing. These compounds should be environmentally friendly and cost-effective. This review presents recent progress within host immunology, vaccinology and a description of novel substances, which have been tested as treatments against ichthyophthiriasis.

Introduction

Ichthyophthirius multifiliis is a protozoan parasite, causing white spot disease and is a major burden for fish farmers and aquarists worldwide. The infective theront stage of the parasite invades the

skin and gills of fish, penetrates the epidermis and settles above the basal lamina [1, 2]. Here it transforms into the trophont stage that feeds on fish tissue until it reaches a size of 0.5-1.0 mm and is macroscopically visible as a white spot [3]. The mature trophont exits the fish and transform into a tomont, which seeks bottom surfaces for encystment into a tomocyst in which asexual reproduction takes place. When the trophont emerges from the fish host it disrupts the epidermis and gill epithelia, which may challenge osmoregulation and leave the fish susceptible to secondary infections [4, 5]. High mortalities occur during epidemics and the fish die from either theront penetration, parasites feeding on cells and tissues or trophont escape from the fish surfaces.

At least five different serotypes of this parasite exist, characterized by differences in the surface immobilisation antigens (Iag) [6] and more serotypes may exist since a total of 17 Iag-genes have been identified in the *I. multifiliis* genome [7]. These Iags are immuno-dominant and are thereby targeted by *I. multifiliis*-specific antibodies produced in the host [8]. Cross-linking of Iags by antibodies elicits either an escape response in the parasites or immobilization [9, 10]. The immunological responses of the hosts have been investigated since 1910 [11] and both innate and adaptive factors are activated during infection [3, 8]. Immunoglobulins are of particular importance in the protective response at mucosal surfaces [8, 10] but a more detailed description of the immune mechanisms is needed.

Different treatments have been used to combat the parasite with treatment regimes changing according to new legislation regarding toxicity and carcinogenicity of the applied substances. The trophont stage of the parasite, which is protected by the epidermis of the fish, is generally more resistant to treatments than the theront stage but it takes intensive effort and repetitive treatments to eliminate the infection by targeting the theront stage. A search for novel efficient and safe compounds for the treatment of white spot disease is ongoing and some new drug candidates appear promising. Prophylaxis is in the long run cheaper and environmentally safer than treatments and management methods including water filtration technologies [12] and immunoprophylactic measures [13, 14] may be part of a future integrated control system. Due to the well described protective immune response erected by fish against *I. multifiliis* a continuous search for vaccines is being undertaken. Progress within this field is evident however, no vaccine has yet been developed that targets all serotypes and is produced independently of parasite production.

Novel investigations have utilized the zebrafish as a model organism to further investigate the immunological mechanisms involved in innate and adaptive responses of the host [15-17]. This model holds the potential for further investigation of the *in vivo* immunological mechanism involved in the responses in an unprecedented way, due to the availability of transparent and immunologically relevant transgenic lines. Utilising this model may provide a more detailed description of host/parasite interactions and key factors for production of vaccines may be revealed.

This review is focused on the current knowledge and understanding of immunology, vaccinology and treatments and building on information gathered into earlier reviews [3, 4, 8].

Immunological responses

Historically, immune response studies against *I. multifiliis* have been conducted primarily with rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*)). It has been assumed that the responses are similar across species yet this is not always the case. For example, channel catfish do not possess IgT immunoglobulins (an immunological important mucosal antibody in rainbow trout) corresponding to IgZ in zebrafish. The skin of channel catfish has antibody-secreting cells for IgM instead and IgD is a prominent Ig in this species. Antibody titers increase from 4 h post immunisation with *I. multifiliis* [18] and species-specific responses will differ due to species related characteristics, illustrating the importance of conducting these investigations at the species level. Therefore, this review divides current knowledge into primary and secondary immunological responses according to findings at the species level.

Recently, investigations have expanded to other species of fish and new tools for immunological analyses have been applied, which contribute to the understanding of the complicated host/parasite interactions.

Primary response

Rainbow trout

Non-specific innate factors target the parasite, which was shown by testing cross protective responses across different fish pathogens and *I. multifiliis* or the *in vitro* demonstration of

serological cross-reactivity between *I. multifilis* and *Tetrahymena pyriformis* [19-21]. Complement factors have also been suggested to be involved in the innate response against *I. multifiliis* [13, 21-23] and evidence that C3 plays an important role, together with the mucosal immunoglobulin IgT, in the fight against the parasite in the skin has recently been provided [24].

Cellular factors have been shown to be involved in the primary response. Four days following infection, MHCII+ cells with the morphology of macrophages and CD8α+ cells were found to surround the parasites in the gills [23]. Gene expression of the cytokines IFN-gamma (which activates macrophages) was elevated both during the innate and the adaptive response indicating that macrophages may play a key role in both responses [23]. The production of serum amyloid a (SAA) is highly upregulated during *I. multifiliis* infections [13, 22, 25] but the function of this apolipoprotein in relation to ichthyophthiriasis is unknown.

A study focusing on primary immune responses was conducted on rainbow trout larvae receiving an infection with *I. multifiliis*. IL-1 beta was the first gene to be up-regulated, followed by IL-8, cathelicidin-2, TNF-alfa, hepcidin, IL-6, iNOS and SAA. MHCII and SAA were found at the sites of parasite localization. This study showed that 10 day old larvae are able to regulate immune-relevant factors during an *I. multifiliis* infection [26].

Channel catfish

It is known that toll-like receptors (TLRs), which are type-I integral trans-membrane receptors and crucial for recognition of different pathogen associated molecular patterns (PAMPs), are activated during parasite infections. They are implicated in innate sensing of the parasites in mammals [27], however their functions are still relatively poorly understood in fish. During an *I. multifiliis* infections in channel catfish (*Ictalurus punctatus*) it was found that the TLRs 1, 2, 9, 19 were upregulated in skin and TLR2, 9, 19 in gill and that the TLRs 21 and 25 were upregulated in spleen and kidney [28]. Immune-relevant gene expression has also been studied in channel catfish immunised intra-peritoneally (i.p.) with live theronts and already from 4 h to 2 days post-immunisation expression of IgM, IgD, TLRs, complement factors and a range of cytokines increased. Immune cell receptor genes for CD4, Cd8α, MHC I, MHCIIβ, TcRα and TcRβ were upregulated from 4 h to 6 days post-immunisation, indicating activation of cell mediated responses [18]. Nonspecific cytotoxic cells (NCC) have also been suggested to play a role in innate immune responses of channel catfish against the parasite [29, 30].

123

124 *Carp*

125 Bauer (1954) characterized the immune response in carp against *I. multifiliis* and reported that the
126 protective response was dependent on the immunization dosage. In this species the cell-mediated,
127 innate immune response has been investigated showing that penetration of the skin by the parasite
128 elicits chemokine release [31] and within 24 h neutrophils are attracted to areas of infection [32].
129 The neutrophils surround the parasites during the next 2-6 days and thereafter other inflammatory
130 cells such as eosinophils and basophils become attracted [32-34].

131

132 *Grass carp (*Ctenopharyngodon idellus*)*

133 The major histocompatibility (MH) DAB gene belongs to antigen-presented genes in the class II
134 genomic region and is associated with anti-parasitic resistance. It has been shown that up-
135 regulation of the DAB gene occurs in skin and gills following a primary infection. These results
136 support the long-established opinion that antibody production is involved in responses against *I.*
137 *multifiliis* [35]. Two important signalling molecules involved in TLR signal transduction, TRAF6 and
138 TAK1, were found to be upregulated in grass carp in skin, gill, head kidney, and spleen following an
139 *I. multifiliis* infection [36]. These studies support the notion that TLRs are involved in primary
140 recognition of the parasite.

141

142 *Zebrafish*

143 It has recently been discovered that zebrafish (*Danio rerio*) are susceptible towards the parasite and
144 that they are able to acquire immunity against it. An increase of mucous cells was associated with
145 infected areas and severe proliferation in the gill tissue was observed during a primary infection,
146 with 87.5 % survival compared to 100 % mortality in control fish during a secondary infection [15].
147 Neutrophils, which are key players during innate immunity have been shown to be involved in the
148 response against *I. multifiliis* in carp (*Cyprinus carpio*) [32]. A recent study showed a 3.4 fold
149 increase of neutrophils in the caudal fin 24 h following a primary infection of zebrafish using the
150 transgenic line Tg(MPO:GFP)^{i114}, indicating a direct interaction of the neutrophils and the parasites
151 [16]. During the following two days, the number of neutrophils in the infected area decreased in
152 spite of the parasites increasing in size and whether this is due to other immune cells taking over or
153 evasion by the parasite needs further investigation. However, it was observed that the parasites

ingested moving functional neutrophils, which must be considered a direct host evasion mechanism. By ingesting immune cells signal pathways and immune reactions may be suppressed. The gills of infected zebrafish have also been investigated in an gene expression study revealing that the cytokines IL-1beta, IL-10, TNF-alpha, IL-8, TGF-beta and IL-4/13, the acute-phase reactant SAA, the compliment factor C3 and a neutrophil marker were up-regulated in infected fish compared to non-infected fish, indicating immune activation [37]. This immune activation is comparable to other fish species infected with this parasite such as rainbow trout [13, 25], carp [31, 38, 39] and channel catfish [18].

Zebrafish are, however, naturally more resistant to *I. multifiliis* infections than rainbow trout, yet a high infection (lethal) can be obtained [40] if zebrafish are exposed to a stressor at the same time e.g. transfer to a new tank, crowding or a decreased quality of water (unpublished results from our laboratory). Zebrafish are also more resilient towards the parasite and obtain much lighter gill infections compared to channel catfish [41]. This points towards zebrafish having innate factors that play a key role in natural protection. The use of genetics approaches in this species is highly relevant and holds promise for an elucidation of host factors responsible for susceptibility and resistance.

Secondary response

It has been known since 1989 that a glycophosphatidylinositol (GPI) anchored protein, the immobilisation antigen (Iag) is a target for protective antibodies of the host [42, 43]. The GPI is a common PAMP amongst protozoans and may be the dominant glycolipid coating the surface of protozoan parasites [27]. *I. multifiliis* specific antibodies cross-bind the Iags and the parasite either escapes the immune fish or gets immobilized and destroyed. A few parasites may settle and develop normally. Even though it is recognized that immunoglobulins are key players for protection against *I. multifiliis* in immune fish it has also been found that a cell-mediated immune response is involved [44].

Rainbow trout

Since 1910 it has been recognised that fish may acquire protection against *I. multifiliis* following sub-lethal infections [45]. Rainbow trout also acquires protection against the parasite following sub-lethal infections or intraperitoneal injections with live parasites [13, 46]. In this species, it was

185 discovered that the mucosal immunoglobulin IgT and IgM bound directly to the parasite in the gills
186 of immune fish [9] within two hours following infection. In carp it has been shown that the
187 parasites exit prematurely the immune fish, within two hours [47], and if this also is the case in
188 rainbow trout, then humoral factors may be playing the major role in protection.
189 Since the discovery of the specialized mucosal immunoglobulin IgT in rainbow trout [48] its
190 functionality has been investigated in skin, gill and gut infections [49-51]. In rainbow trout IgT plays
191 a major role in the protective response against *I. multifiliis* at the mucosal surfaces skin and gills. In
192 the skin IgT^+ B cells represent the majority of the B cell subtypes and during infection with *I.*
193 *multifiliis* the parasite is covered with IgT. Fish surviving an infection had high concentrations of IgT
194 in the mucus [49]. In the gills IgT^+ B cells were, for the first time, shown to proliferate locally and
195 generate *I. multifiliis* specific IgT. IgT coated the parasite and, in contrast to a previous study [9],
196 almost no IgM was found to bind the parasite in the gills [50]. *I. multifiliis* specific IgM was detected
197 in the serum of infected and survivor fish [49, 50]. Preliminary results from our laboratory show
198 that transcripts of IgM in the gills are 10 fold upregulated in immune rainbow trout whereas IgT is 7
199 fold upregulated 1 h following challenge. This could indicate that IgM^+ B memory cells reside in the
200 gill tissue or that they are homing to areas of infection during a challenge.
201 Markers of cells associated with the cellular adaptive response such as IgM, IgT, MHCII and
202 CD8 are regulated in areas of infection [23] but their direct involvement during an adaptive
203 response needs further investigation.
204
205 *Channel catfish*
206 Protection against *I. multifiliis* by immunization in channel catfish was first demonstrated by Goven
207 et al. 1980 [52]. Tetrameric IgM, which together with IgD are the only classes of immunoglobulins
208 present in channel catfish, is the functional protective antibody produced in the skin and in the
209 systemic compartment of channel catfish [8, 53-55]. Using this species it was discovered that the
210 parasite was forced to exit the host after administration of mouse IgG antibodies targeted against
211 the immune-dominant surface protein Iag, confirming the role of Iags in protective immunity [10].
212 In channel catfish, it has been shown that three years following an *I. multifiliis* infection IgM^+
213 memory B cells maintained humoral immunity by differentiating into antibody secreting cells during
214 a challenge infection, and were found both in systemic and mucosal tissue compartments [56].
215

Carp

Bauer (1953) demonstrated a protective response in carp dependent on the primary infection pressure. Hines and Spira (1974) demonstrated that serum from immunized fish was able to immobilise parasites *in vitro* [57]. In this species, it was shown that only around 5% of theronts were able to infect immunized fish [32, 47]. The remaining theronts prematurely exited the host within two hours. The premature exit was so fast that it can be doubted that the protective mechanisms rely on cell-mediated responses [47]. Established parasites in immune fish were surrounded by neutrophils, macrophages, eosinophilic granular cells (ECG) and basophils, which did not affect the parasite [33].

Vaccines

Since fish are able to acquire protection against *I. multifiliis* following exposure to the parasite, vaccination as a prophylactic approach represents a better alternative than repeated treatments to control the disease. With a vaccine, handling of and stress for the fish is minimized and given that the fish will not suffer from the diseases, there will be a major impact on the welfare of the animal. A further benefit of vaccines, is the reduced impact of repetitive treatments of environmentally disruptive substances.

Sub-lethal infections and i.p. injections provide fish hosts with protective immunity [9, 11, 13, 58-61], which holds promise for possible vaccines. However, to date there is no commercially available prophylaxis on the market. Immunizations using dead parasite materials have induced varying degrees of protection and Burkart *et al.* (1990) induced a significant level of protective immunity in channel catfish using formalin-killed trophonts [62]. In rainbow trout fry protection was also obtained by i.p. injection using sonicated formalin-killed trophonts [63]. On the other hand, Xu *et al.* (2009) found that sonicated trophonts, and not formalin inactivated trophonts, induced both serum and cutaneous antibody responses and protection against a live theront challenge in channel catfish [64]. Vaccinations with purified Iag and Freund's complete adjuvant induce protection only against the same serotype of the parasite bearing homologous Iag on their surface [65, 66], however other protective proteins exist because vaccination with live theronts, either as natural sub-lethal infections or i.p. injections, confers protection which is not serotype specific [67, 68].

I. multifiliis is an obligate parasite and culturing of the parasite without the use of fish has, to date, not been successful. Therefore, it is impossible to produce parasites in bulk for the large-scale vaccine production that would be needed to make a parasite-based vaccine. Therefore, a biotechnological approach is more feasible.

DNA vaccines using an IAG52B as a vaccine candidate were successfully expressed in rainbow trout and channel catfish muscle tissues, inducing Iag-specific antibody responses but not providing significant protection [14, 69].

A recombinant vaccine using *Escherichia coli* with a successfully expressed fusion protein with a 316 bp gene fragment containing a potential antigenic epitope of a 48 kDa Iag induced 95 % survival in goldfish compared to 55 % for the controls. Even though the result was promising no further studies have been reported for this recombinant vaccine. The gene for Iag (IAG52B) has been inserted into the closely related ciliate *Tetrahymena thermophila* and successful recombinant expression was achieved [70, 71] and protection in channel catfish was obtained. However, the concentration of cadmium, which was the gene promoter in the transformed *T. thermophila* was found to be elevated in fish tissues [72], thus posing a potential health danger.

A radiovaccine (gamma irradiated trophonts) has also been tested in rainbow trout using feed and bath administration and immune parameters were activated but no data on protection was reported [73, 74].

A live recombinant *Lactococcus lactis* vaccine expressing a 48 kDa immobilization antigen has been tested in goldfish. The recombinant vaccine was administered through feed and, even though Iag-specific antibody levels were detected, only a limited effect was observed on survival of the fish (50-60 % compared to 40 % in controls) [75].

Progress in genomic and transcriptomic techniques have opened up the door for identification of novel vaccine and drug targets, with a recent study using *in silico* techniques to select new antigens from the proteome for potential immunogenicity. Three antigens were chosen, expressed recombinantly and tested in an adjuvanted sub-unit vaccine against *I. multifiliis*. Sonicated tomonts were used as benchmark and the sub-unit vaccine performed almost as efficient (modest protection) as the benchmark vaccine [76]. Such biotechnology-based approaches are viewed increasingly as promising for the development of vaccines against this parasite, which cannot be cultivated in the laboratory without sacrificing fish.

Explorative platform and future perspectives

With advances in biotechnological approaches a vaccine against this important fish pathogen is within reach. Two major obstacles have to be overcome: 1) the expression system must be inexpensive for large scale productions and must produce recombinant proteins with the correct tertiary structure for immunogenicity, 2) novel antigens, which are protective against all serotypes are necessary requirements. Immunological studies are essential to understand how the fish is able to battle the parasite and thus may help us in the search for an efficient vaccine strategy or novel antigens.

With the zebrafish available as a novel explorative platform for this fish disease unprecedented opportunities are offered. The underlying host mechanisms behind the immunological responses can be mapped in much more detail than previously possible and with immunologically relevant transgenic lines specific aspects of the response can be investigated. An example would be the study of the impact of neutrophils in disease development, using a line with a normal level of neutrophils, to compare pathology with a line where all neutrophils are depleted during infection. Novel methods have been developed for *in vivo* studies of host/parasite interactions using specialized equipment such as a confocal microscope [16]. Since the parasite is naturally restricted to gills and skin these observations can be conducted with non-invasive techniques.

Until a cost-efficient vaccine against *I. multifiliis* is on the market, management and treatment are the only means of controlling the disease. Using zebrafish for treatment studies provides significant advances as *in vitro* and *in vivo* studies can be conducted according to common practice. Both acute and lethal toxicity estimations can be obtained easily according to the OECD guidelines for fish embryo acute toxicity test (test no. 236) [77]. Zebrafish are furthermore relatively easy to keep and breed in captivity with a single breeding pair able to produce up to 300 eggs per week.

I. multifiliis is still a major threat to sustainable aquaculture and for aquarists and an effective means of control is urgently needed. Until an effective prophylaxis has been developed, repetitive treatments are required. However, revealing more detail of the immunological mechanisms involved in the fight against the parasite may provide knowledge leading to novel approaches to combat the disease.

Treatments

At present, there is no vaccine on the market against ichthyophthiriasis and the only way to control the parasite is through treatments of fish or water containing infective theronts. Through the years, the primary treatment against *I. multifiliis* has changed according to efficacy, toxicity or carcinogenicity of the substances. Some treatments are non-toxic and non-carcinogenic e.g. salt, pH and temperature regulations and may be more or less effective. Salt has been used against *I. multifiliis* since 1893 [4, 78, 79] and in 1972 Cross [80] suggested that salt only had a positive effect on fish recovery. Since then, several studies have shown that salt can directly affect and reduce parasite populations [81-85]. Both the parasite and the fish can be severely affected by pH and considerations related to fish species and welfare have to be taken into account. It has been shown that the parasite thrived at pH 7 in silver catfish (*Rhamdia quelen*) and reduction or increase of pH reduced parasite survival [86-88]. Temperature is a common way of controlling ichthyophthiriasis for hobby aquarists and in this forum, it is said that raising the temperature to 30 C° "burns" out the parasite. It has been shown that raising the temperature to 32 C° for 5 days does, indeed, destroy the parasite but this has limited application for production in large-scale aquaculture [4].

The use of chemicals for treatment of white spot disease is common practice for aquaculturists and aquarists. Aquarists use, primarily, formulations that include malachite green, methylene blue, copper sulfate and formaldehyde. Malachite green and methylene blue target both the free-living stages and the stages within the fish epidermis, which indicate that the dyes penetrate deeply or act systemically and kill subcutaneous stages of *I. multifiliis* [89]. However, reports of carcinogenic and teratogenic effects of malachite green on humans [90-93] led to the withdrawal of the substance for food fish in the USA and in Europe [4]. Methylene blue is an aniline dye and is not likely to get approved for food fish. Copper sulfate is also effective against ichthyophthiriasis but only prevents transmission and can be extremely toxic to fish in water of low alkalinity [89, 94]. It is not approved for use in fish for consumption. The use of formaldehyde for food fish is described in the following paragraph.

Auxiliary products for water treatment

Control can be achieved by use of water treatment with auxiliary substances, which target free-living stages of the parasite in the fish water, preventing infection of fish. For many years formaldehyde has been the most used substance in the aquaculture industry worldwide to combat

I. multifiliis [4, 95] and this chemical is lethal to the parasites at 50-100 mg/L between 30-60 min. Such dosages can be tolerated by certain fish species e.g. rainbow trout [12, 96]. The toxicity and effectivity of treatments are also dependent on water parameters such as temperature, pH and hardness [97] and should, therefore, be designed to match individual aquaculture systems. Furthermore, formaldehyde has been classified as carcinogenic (EU directive 2004/37/EC) [98] and in Denmark legislation declares that formaldehyde only can be used when there is no effective substitute for it [99]. A series of other environmentally problematic compounds have also recently been suggested for water treatment. One study focused on *in vitro* treatments using chlorine dioxide against *I. multifiliis* and *in vivo* studies in infected silver catfish (*Rhamdia quelen*). Theronts were killed *in vitro* with 50 mg/L for 1 h and with a dosage of 25 mg/L for 48 h the parasite burden *in vivo* was reduced by more than 50 % [100]. The use of copper sulfate and chloramine T to kill theronts was found to be effective at a concentration of 1 mg/L and 21 mg/L for 13 min, respectively but the toxicity was highly dependent on water parameters [101]. Nitazoxanide has also been tested in silver catfish juveniles against the parasite and the use of 1.5 mg/L showed an increased survival of 97 % compared to 40 % survival in the controls and a reduction of the parasite burden with 98 % [102].

Table 1. A selection of recently *in vitro* tested substances against *I. multifiliis*. Concentrations of the substances, theront and tomont mortalities and references are provided.

Table 2. A selection of recently *in vivo* tested substances against *I. multifiliis.* The relevant fish species, the LC_{50}, concentrations of the substances, percent mortality of treated versus control animals, the reduced parasite burden and references are provided.

Environmentally friendly products leave only highly biodegradable products in the fish farm. Among these, compounds based on the release of hydrogen peroxide have been introduced with some success during recent years. Hydrogen peroxide has been tested and kills the parasites with a lower concentration compared to formaldehyde [12]. It is lethal for the parasites at concentrations which are tolerable for the fish. Peracetic acid, which disintegrates to hydrogen peroxide and acetic acid (which further disintegrates to water, oxygen and carbon dioxide) has been tested through continuous addition in a field trial study against *I. multifiliis* and was found to be efficient in a

concentration of 0.10-0.15 mg/L [103, 104]. Another effective auxiliary compound applied at farm level is sodium percarbonate, which is known to eliminate theronts within hours [105].

Drugs

Antiparasitic drugs, such as toltrazuril, which are not specifically licensed for use in fish and are used in other animals, can be used in the European community if the so-called "cascade principle" is taken into account. This regulation is described in EC Directive 2004/28/CE and states that drugs licensed for other host animals can be used if no drug is available for treatment of a disease in the specific species [106]. Toltrazuril was tested for any protective effect against *I. multifiliis* through feed administration and it was found that the drug conferred protection against infection but had no effect on already established parasites [107]. Antibiotics, such as this one, are not seen as acceptable for control measures due to their negative impact on the environment (see also the section Actinobacteria, for more detail).

There is a standing need for novel agents to kill *I. multifiliis* and in recent years many substances have been investigated. To assess a novel substance a range of investigations should be reviewed to get a holistic and realistic picture of the usefulness of the agent, including: 1) *in vitro* studies describing the effect on theronts, non-encysted tomonts and encysted tomonts. Concentration, time span and mortality should be registered, 2) *in vivo* studies conducted on fish with a primary infection. Concentration, time span for reduced mortality and parasite burden should be registered, 3) the median lethal concentration (LC_{50}) of the substance determined for the relevant fish species.

A good candidate substance will kill all stages of the parasite at a low concentration *in vitro*, significantly reduce mortality and the parasite burden at a low concentration *in vivo* and have an LC_{50} value for the relevant fish species much higher than the needed concentration for treatment against ichthyophthiriasis.

It should be emphasised that any compound being considered as a commercial drug must hold the promise of cost-efficient, large-scale production and regulatory compliance for both fish and humans.

Plant extracts

399 Plant extracts have also been suggested as alternative treatments against *I. multifiliis*. A series of
400 publications have focused on antiparasitic effects but environmental and toxic effects need to be
401 addressed as well. The effect of various of these plant extracts is described below and a summary
402 of the related *in vitro* and *in vivo* investigations can be found in Tables 1 and 2.
403 Three essential oils from *Melaleuca alternifolia*, *Lavandula angustifolia* and *Mentha piperita* were
404 tested for their toxic effect on *I. multifiliis* trophonts obtained from pacu (*Piaractus*
405 *mesopotamicus*). All three oils showed toxicity towards the trophonts and *M. alternifolia*, was also
406 tested as an *in vivo* antiparasitic agent in infected fish, showing some promise at relatively high
407 concentrations compared to other substances described in this review [108]. Ethanol extracts from
408 three medicinal plants *Cynanchum atratum*, *Zingiber officinale*, and *Cynanchum paniculatum* were
409 tested against theronts, nonencysted tomonts and encysted tomonts and administered, through
410 feed, against *in vivo* infections in grass carp. A concentration of 8 mg/L for *Z. officinale* was 100 %
411 effective against theronts and a concentration of 4 mg/L of *C. atratum* was sufficient to kill
412 encysted tomonts. Applying the extract to the feed had a limited effect [109]. Another study
413 showed that cynatratoside-C isolated from *C. atratum* showed 100 % mortality against *I. multifiliis*
414 theronts from grass carp in approximately three hours at a low concentration of 0.25 mg/L. The
415 same concentration was used for *in vivo* infections and showed significant treatment efficacy and
416 protection. Furthermore, the substance was tested for toxicity to grass carp and the median lethal
417 concentration (LC_{50}) was found to be 46.8 mg/L and thus safe for treatment against *I. multifiliis*
418 [110].
419 Gracillin and zingibernsis newsaponin from the plant species *Costus speciosus* showed
420 efficiency against *I. multifiliis in vitro* and *in vivo* in grass carp. *In vitro* concentrations of 0.8 and 4.5
421 mg/L of Gracillin and zingibernsis newsaponin, respectively were 100% lethal to theronts whereas a
422 concentration of 1 and 5 mg/L was effective against nonencysted tomonts and encysted tomonts
423 respectively after 6 h exposure. *In vivo* experiments demonstrated that the latter concentrations
424 were also effective *in vivo* and that the parasite burden was significantly reduced. Acute toxicities
425 (LC_{50}) of gracillin and zingibernsis newsaponin to grass carp were 1.64 and 20.7 mg/L, respectively
426 [111].
427 Root bark of white mulberry (*Morus alba)* has also been investigated for its potentially anti-
428 parasitic effect against *I. multifiliis*. One study used a range of extraction methods on powdered
429 bark, and *in vitro* efficiency was shown but the concentrations were relatively high compared to the

other tested substances described in this review [112] (Table 1). In another study, two flavonoids (kuwanons G and O) induced 100 % mortality in theronts at a concentration of 2 mg/L and were able to reduce infectivity of theronts following exposure. The median lethal concentrations (LC_{50}) of kuwanons G and O to grass carp were 38.0 and 26.9 mg/L respectively [113].

Extracts of chelerythrine and chloroxylonine from *Toddalia asiatica* were 100% effective against *I. multifiliis* in concentrations of 1.2 mg/L and 3.5 mg/L *in vitro*, respectively, within 4 h. *In vivo* experiments showed that infected fish treated with chelerythrine and chloroxylonine at the concentrations of 1.8 and 8.0 mg/L, respectively carried significantly fewer parasites. The acute toxicity (LC_{50}) of chelerythrine for goldfish was 3.3 mg/L [114]. The compound pentagalloylglucose extracted from *Galla chinensis* was also shown to be lethal to *I. multifiliis* with theront mortality at 100 % after treatment with 2.5-20 mg/L for 6-234 min. Infectivity of theronts was significantly reduced at 1, 2 and 5 mg/L and pentagalloylglucose at 20 mg/L was effective in treating infected catfish and preventing naive catfish from *I. multifiliis* infection. The LC_{50} for this compound in channel catfish was 151.3 mg/L [115].

Two compounds (psoralidin and isopsoralen) extracted with methanol from *Psoralea corylifolia* were also tested *in vitro* and *in vivo* against *I. multifiliis* in goldfish (*Carassius auratus*). Psoralidin was found to be the most effective of them, killing all theronts *in vitro* at a concentration of 0.8 mg/L during a 4 h exposure and terminated reproduction in trophonts at 0.9 mg/L and in encysted tomonts at 1.2 mg/L. Infection pressure was significantly reduced after exposure of 2.5 mg/L psoralidin within 5 h in *in vivo* trials [116]. Another study with methanol extracts from the same plant showed 100 % theront mortality at 1.25 mg/L within 4 h, and 100 % mortality of non-encysted tomonts and terminated reproduction of encysted tomonts at 1.2 mg/L within 6 h. A 24 h bath of infected goldfish in 5 mg/L significantly reduced survival and reproduction of *I. multifiliis* [117].

Actinobacteria

Extracellular fungicidal products from *Streptomyces* have been shown to have some effect against *I. multifiliis* and within recent years isolated compound have been tested for their bioactivity. Amphotericin B from *Streptomyces* sp. strain HL-2-14 has been tested for *in vitro* and *in vivo* efficacy against *I. multifiliis* in grass carp. The *in vitro* studies showed that a median lethal concentration (LC_{50}) of 0.8 mg/L and 4.3 mg/L killed theronts and non-encysted tomonts within 30

min and 2h respectively. The related *in vivo* studies showed that 5 mg/L significantly reduced *I. multifiliis* infectivity and intensity on grass carp but also found that the acute toxicity (LC_{50}) of amphotericin B on grass carp was 20.6 mg/L, with fish mortality observed when exposed to 13 mg/L [118]. The compound nystatin from *Streptomyces griseus* SDX-4 was effective against theronts and encysted tomonts at a concentration of 6 mg/L for 4 h. The same concentration *in vivo* reduced the mortality of goldfish and both parasite survival and reproduction were significantly reduced. The median lethal dose (LD_{50}) of nystatin for goldfish was 16.8 mg/L [119].

Many of the substances mentioned here have antibiotic properties and consequently are less desirable as control measures against *I. multifiliis* since they may lead to environmental selection of antibiotic-resistant microorganisms. Some of the same antibiotics used, or with potential for water treatment in aquaculture, are also used to treat human disease and the microorganisms causing such disease may increase in resistance to these antibiotics as a result of their use in fish farms. For this reason, and given that compounds may only be degraded very slowly in nature, the use of antibiotics as a control measure in aquaculture may create public health issues and is unlikely to be widely adopted.

Biological control strategies

A feasible approach to control ichthyophthiriasis could be the implementation of biological control. In warm water systems the use of an algae grazing fish, the leopard pleco (*Glyptoperichthys gibbiceps*), resulted in significant reduction of *I. multifiliis* infections as the fish graze the biofilms on which the parasite settles for encystment [120]. Copepods and other filtrating microorganisms are frequently found in fish tank systems and they may influence the biota in the system. It was found that cyclopoid copepods ingested oncomiracidia of *Pseudodactylogyrus* spp [121]. Likewise, a variety of filter feeders may be able to ingest tomonts and theronts and thereby reduce infection pressure of *I. multifiliis* in the system. Such biological control systems may be considered as relatively benign, in environmental terms and may benefit controlling the disease.

491 Table 1. A selection of recently *in vitro* tested substances against *I. multifiliis*.

In vitro test	Origin	Compound	Conc. mg/L (exposure time)	Theront mortality	Conc. mg/L (exp. time)	% non-encysted tomont mortality	Conc. mg/L (exp. time)	% encysted tomont mortality	References
Chemicals	Chloride dioxide		50 (1 h)	100					[100]
	Copper sulfate*		1 (13 min)	100	-	-	-	-	[101]
	Chloramine T*		21 (13 min)	100	-	-	-	-	[101]
	Nitazoxanide								[102]
Plant extracts	*Melaleuca alternifolia*	Essential oil	-	-	114 µL/L (4 h)	93.32	-	-	[108]
	*Lavandula angustifolia**	Essential oil	-	-	114 µL/L (4 h)	73.28	-	-	[108]
	*Mentha piperita**	Essential oil	-	-	114 µL/L (4 h)	84.34	-	-	[108]
	Cynanchum paniculatum	Ethanol extract	16 (4 h)	100	4 (5 h)	45.5	16 (-)	100	[109]
	Zingiber officinale	Ethanol extract	8 (4 h)	100	4 (5 h)	35.6	16 (-)	100	[109]
	Cynanchum atratum	Ethanol extract	16 (4 h)	100	4 (5 h)	78.3	4 (-)	100	[109]
		Cynatratoside-C	0.25 (186.7 +-5.8 min)	100	-	-	-	-	[110]
	Costus speciosus	Gracillin	0.8 (4 h)	100	0.8 (6 h)	100	1 (6 h)	100	[111]
		Zingibernsis	4.5 (4 h)	100	5 (6 h)	100	5 (6 h)	100	[111]
	Morus alba	Acetone extract (powdered bark)	8 (4 h)	100	25 (4 h)	100	50 (4 h)	100	[112]
		Kuwanon G	2	100	-	-	-	-	[113]

		Kuwanon O	2	100	-	-	-	-	[113]
	Toddalia asiatica	Chelerythrine	-	-	1.2 (-)	100	-	-	[114]
		Chloroxylonine	-	-	3.5 (-)	100	-	-	[114]
	Galla chinensis	Pentagalloylglucose	2.5-20 (5.6-233.9 min)	100	80 (4 h)	100	-	-	[115]
	Psoralea corylifolia	Psoralidin	0.8 (4 h)	100	0.9 (6 h)	100	1.2 (6 h)	Terminate reproduction	[116]
		Methanol extract*	1.25 (4 h)	100	5 (4 h)	100	5 (4 h)	88.9	[117]
Actinobacteria	Streptomyces sp. strain HL-2-14	Amphotericin B	1.6 (30 min)	85.4	12.5 (2 h)	87.6	-	-	[118]
	Streptomyces griseus SDX-4	Nystatin	6 (4 h)	100	-	-	6 (4 h)	100	[119]

*No information available for these substances in Table 2. Conc. = concentration, exp. = exposure time

Table 2. A selection of recently *in vivo* tested substances against *I. multifiliis.*

In vivo test	Origin	Compound	Fish species	LC_{50} mg/L	Concentration mg/L	% mortality, treated/contr.	% reduced parasite burden	References
Chemicals	Chloride dioxide		Silver catfish	-	25 (48 h bath)		>50	[100]
	Nitazoxanide		Silver catfish	-	1.5	10/60	98 % with 1.5 mg/L	[102]
Plant extracts	*Melaleuca alternifolia*	Essential oil	Pacu	-	50 µL/L (bath)	47/100	99	[108]
	Cynanchum paniculatum	Ethanol extract	Grass carp	-	4 % (feed, 10 days)	0/0	18.6 (non significant)	[109]

	Zingiber officinale	Ethanol extract	Grass carp	-	4 % (feed, 3 days)	0/0	51.6 (significant)	[109]
	Cynanchum atratum	Ethanol extract	Grass carp	-	4 % (feed, 10 days)	0/0	76.5 (significant)	[109]
		Cynatratoside-C	Grass carp	46.8	1 (10 days bath)	0/100 (after 30 days)	100 (after 15 days)	[110]
	Costus speciosus	Gracillin	Grass carp	1.64	1 (10 days bath)	10/100 (after 15 days)	-	[111]
		Zingibernsis	Grass carp	20.7	5 (10 days bath)	6.7/100 (after 15 days)	16.7	[111]
	Morus alba	Acetone extract (powdered bark)	Grass carp	80		-	-	[112]
		Kuwanon G	Grass carp	38	-	-	-	[113]
		Kuwanon O	Grass carp	26.9	-	-	-	[113]
	Toddalia asiatica	Chelerythrine	Goldfish	3.3	1.8	0/20	55.4	[114]
		Chloroxylonine	Goldfish	-	8	0/20	45.9	[114]
	Galla chinensis	Pentagalloylglucose	Channel catfish	151.3	20 (10 days bath)	6.7/100	-	[115]
	Psoralea corylifolia	Psoralidin	Goldfish	5.6	-	-	-	[116]
Actinobacteria	Streptomyces sp. strain HL-2-14	Amphotericin B	Grass carp	20.6	5 (bath)	30/100	53.3	[118]
	Streptomyces griseus SDX-4	Nystatin	Goldfish	16.8	6 (bath day 1, 3 and 5 post infection)	23.8/85.7	45.7	[119]

Contr. = control

Acknowledgements

This work was funded by H2020 Food, Grant/Award Number: 634429

References:

[1] M. Ewing, K. Kocan, S. Ewing, *Ichthyophthirius multifiliis* (Ciliophora) invasion of gill epithelium, Journal of Protozoology 32(2) (1985) 305-310.

[2] M. Ventura, I. Paperna, Histopathology of Ichthyophthirius-multifiliis infections in fishes, Journal of Fish Biology 27(2) (1985) 185-203.

[3] K. Buchmann, J. Sigh, C.V. Nielsen, M. Dalgaard, Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*, Vet Parasitol 100(1-2) (2001) 105-16.

[4] R.A. Matthews, *Ichthyophthirius multifiliis* Fouquet and Ichthyophthiriosis in Freshwater Teleosts, Adv Parasitol 59 (2005) 159-241.

[5] M. Ewing, K. Kocan, Invasion and development strategies of *Ichthyophthirius-multifiliis*, a parasitic ciliate of fish, Parasitology Today 8(6) (1992) 204-208.

[6] H. Dickerson, T. Clark, A. Leff, Serotypic variation among isolates of *Ichthyophthirius-multifiliis* based on immobilization, Journal of Eukaryotic Microbiology 40(6) (1993) 816-820.

[7] R. Coyne, L. Hannick, D. Shanmugam, J. Hostetler, D. Brami, V. Joardar, J. Johnson, D. Radune, I. Singh, J. Badger, U. Kumar, M. Saier, Y. Wang, H. Cai, J. Gu, M. Mather, A. Vaidya, D. Wilkes, V. Rajagopalan, D. Asai, C. Pearson, R. Findly, H. Dickerson, M. Wu, C. Martens, Y. Van de Peer, D. Roos, D. Cassidy-Hanley, T. Clark, Comparative genomics of the pathogenic ciliate *Ichthyophthirius multifiliis*, its free-living relatives and a host species provide insights into adoption of a parasitic lifestyle and prospects for disease control, Genome Biology 12(10) (2011).

[8] H.W. Dickerson, R.C. Findly, Immunity to Ichthyophthirius infections in fish: A synopsis, Developmental and Comparative Immunology 43(2) (2014) 290-299.

[9] L. Jorgensen, R. Heinecke, K. Skjodt, K. Rasmussen, K. Buchmann, Experimental evidence for direct *in situ* binding of IgM and IgT to early trophonts of *Ichthyophthirius multifiliis* (Fouquet) in the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases 34(10) (2011) 749-755.

[10] T. Clark, T. Lin, H. Dickerson, Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host, Proceedings of the National Academy of Sciences of the United States of America 93(13) (1996) 6825-6829.

[11] A.L. Buschkiel, Beträge zur kenntnis des *Ichthyophthirius multifiliis* Fouquet., Archiv für Protistenkunde 21 (1910) 61-102.

[12] R. Heinecke, K. Buchmann, Control of *Ichthyophthirius multifiliis* using a combination of water filtration and sodium percarbonate: Dose-response studies, Aquaculture 288(1-2) (2009) 32-35.

[13] L.V. Jorgensen, E. Nemli, R.D. Heinecke, M.K. Raida, K. Buchmann, Immune-relevant genes expressed in rainbow trout following immunisation with a live vaccine against *Ichthyophthirius multifiliis*, Diseases of Aquatic Organisms 80(3) (2008) 189-197.

[14] L. Jorgensen, J. Sigh, P. Kania, L. Holten-Andersen, K. Buchmann, T. Clark, J. Rasmussen, K. Einer-Jensen, N. Lorenzen, Approaches towards DNA Vaccination against a Skin Ciliate Parasite in Fish, Plos One 7(11) (2012).

[15] L.v.G. Jorgensen, Infection and immunity against *Ichthyophthirius multifiliis* in zebrafish (*Danio rerio*), Fish & Shellfish Immunology 57 (2016) 335-339.
[16] L.v.G. Jorgensen, The dynamics of neutrophils in zebrafish (*Danio rerio*) during infection with the parasite *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 55 (2016) 159-164.
[17] T.B. Christoffersen, P.W. Kania, L.v.G. Jørgensen, K. Buchmann, Zebrafish *Danio rerio* as a model to study the immune response against infection with *Ichthyophthirius multifiliis* Journal of Fish Diseases (2016).
[18] D.H. Xu, Q.Z. Zhang, C.A. Shoemaker, D.H. Zhang, G.S.A. Moreira, Molecular immune response of channel catfish immunized with live theronts of *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 54 (2016) 86-92.
[19] B.A. Goven, D.L. Dawe, J.B. Gratzek, *In vitro* demonstration of serological cross-reactivity between *Ichthyophthirius multifilis* Foquet and *Tetrahymena pyriformis* Lwoff, Dev Comp Immunol 5(2) (1981) 283-9.
[20] K. Wolf, M. Markiw, Ichthyophthiriasis -immersion immunisation of rainbow trout (*Salmo-gairdneri*) using *Tetrahymena-thermophila* as a protective immunogen, Canadian Journal of Fisheries and Aquatic Sciences 39(12) (1982) 1722-1725.
[21] K. Buchmann, T. Lindenstrom, J. Sigh, Partial cross protection against *Ichthyophthirius multifiliis* in *Gyrodactylus derjavini* immunized rainbow trout, Journal of Helminthology 73(3) (1999) 189-195.
[22] J. Sigh, T. Lindenstrom, K. Buchmann, The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum), Journal of Fish Diseases 27(7) (2004) 409-417.
[23] M.M. Olsen, P.W. Kania, R.D. Heinecke, K. Skjoedt, K.J. Rasmussen, K. Buchmann, Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: Molecular and immunohistochemical studies, Fish & Shellfish Immunology 30(3) (2011) 859-869.
[24] D. Gomez, Z. Xu, L.V. Jorgensen, K. Buchmann, J.O. Sunyer, Orchestrated interaction between IgT and complement C3 to control a skin parasite of rainbow trout, Fish & Shellfish Immunology 34(6) (2013) 1708-1708.
[25] M.M. Olsen, P.W. Kania, R.D. Heinecke, K. Skjoedt, K.J. Rasmussen, K. Buchmann, Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: Molecular and immunohistochemical studies, Fish & Shellfish Immunology 30(3) (2011) 859-869.
[26] R. Heinecke, K. Buchmann, Inflammatory response of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) larvae against *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 34(2) (2013) 521-528.
[27] D. Ghosh, J.S. Stumhofer, Do you see what I see: Recognition of protozoan parasites by Toll-like receptors, Journal of Immunologi reviews, 2013, pp. 129-40.
[28] F. Zhao, Y.W. Li, H.J. Pan, C.B. Shi, X.C. Luo, A.X. Li, S.Q. Wu, Expression profiles of toll-like receptors in channel catfish (*Ictalurus punctatus*) after infection with *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 35(3) (2013) 993-997.
[29] S. Graves, D. Evans, D. Dawe, Antiprotozoan activity of nonspecific cytotoxic cells (NCC) from the channel catfish (*Ictalurus-punctuates*), Journal of Immunology 134(1) (1985) 78-85.
[30] S. Graves, D. Evans, D. Dawe, Mobilisation and activation of nonspecific city-toxic cells (NCC) in the channel catfish (*Ictalurus punctatus*) infected with *Ichthyophthirius-multifiliis* Comparative Immunology Microbiology and Infectious Diseases 8(1) (1985) 43-51.

[31] S.F. Gonzalez, K. Buchmann, M.E. Nielsen, Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: Inflammatory responses caused by the ectoparasite *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 22(6) (2007) 641-650.
[32] M.L. Cross, R.A. Matthews, Localized leukocyte response to *Ichthyophthirius-multifiliis* establishment in immune carp *Cyprinus-carpio* L, Veterinary Immunology and Immunopathology 38(3-4) (1993) 341-358.
[33] M.L. Cross, Localized cellular-responses to *Ichthyophthirius-multifiliis* - protection or pathogenesis, Parasitology Today 10(9) (1994) 364-368.
[34] R. Hines, D. Spira, Ichthyophthiriasis in mirror carp. 2. Leucocyte response, Journal of Fish Biology 5(4) (1973) 527-534.
[35] H. Yu, Q.G. Yan, Z.B. Wang, Y.J. Lu, M.J. Xu, H. Li, X.Q. Zhu, MH II-DAB gene expression in grass carp *Ctenopharyngodon idella* (Valenciennes) after infection with the ciliate parasite, *Ichthyophthirius multifiliis*, Journal of Fish Diseases 37(1) (2014) 43-50.
[36] F. Zhao, Y.W. Li, H.J. Pan, S.Q. Wu, C.B. Shi, X.C. Luo, A.X. Li, Grass carp (*Ctenopharyngodon idella*) TRAF6 and TAK1: Molecular cloning and expression analysis after *Ichthyophthirius multifiliis* infection, Fish & Shellfish Immunology 34(6) (2013) 1514-1523.
[37] T.B. Christoffersen, P.W. Kania, L.v.G. Jørgensen, K. Buchmann, Zebrafish *Danio rerio* as a model to study the immune response against infection with *Ichthyophthirius multifiliis*, Journal of Fish Diseases 40 (2016) 847-852.
[38] S.F. Gonzalez, K. Buchmann, M.E. Nielsen, Complement expression in common carp (*Cyprinus carpio* L.) during infection with *Ichthyophthirius multifiliis*, Developmental and Comparative Immunology 31(6) (2007) 576-586.
[39] S.F. Gonzalez, K. Buchmann, M.E. Nielsen, *Ichthyophthirius multifiliis* infection induces massive up-regulation of serum amyloid A in carp (*Cyprinus carpio*), Veterinary Immunology and Immunopathology 115(1-2) (2007) 172-178.
[40] L.v.G. Jorgensen, Infection and immunity against *Ichthyophthirius multifiliis* in zebrafish (*Danio rerio*), Fish & Shellfish Immunology 57 (2016) 335-339.
[41] B. Cherry, Laboratory infection of zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctuates*) with the protozoan parasite *Ichthyophthirius multifiliis*: a model for parasite persistence, University of Pennsylvania, 2003. Dissertations available from ProQuest. AAI3109164, http://repository.upenn.edu/dissertations/AAI3109164
[42] H.W. Dickerson, T.G. Clark, R.C. Findly, *Ichthyophthirius multifiliis* has membrane-associated immobilization antigens, Journal of Protozoology 36(2) (1989) 159-164.
[43] T. Clark, Y. Gao, J. Gaertig, X. Wang, G. Cheng, The I-antigens of *Ichthyophthirius multifiliis* are GPI-anchored proteins, Journal of Eukaryotic Microbiology 48(3) (2001) 332-337.
[44] Y.M. Sin, K.H. Ling, T.J. Lam, Cell-mediated immune response of goldfish, *Carassius auratus* (L), to *Ichthyophthirius multifiliis*, Journal of Fish Diseases 19(1) (1996) 1-7.
[45] A.L. Buschkiel, Beträge zur kenntnis des *Ichthyophthirius multifiliis* Fouquet, Archiv für Protistenkunde 21 (1910) 61-102.
[46] J. Sigh, K. Buchmann, Comparison of immobilization assays and enzyme linked immunosorbent assays for detection of rainbow trout antibody-titres against *Ichthyophthirius multifiliis* Fouquet, 1876, Journal of Fish Diseases 24(1) (2001) 49-51.
[47] M.L. Cross, R.A. Matthews, Ichthyophthiriasis in carp, *Cyprinus-carpio* L - fate of parasites in immunized fish, Journal of Fish Diseases 15(6) (1992) 497-505.
[48] J.D. Hansen, E.D. Landis, R.B. Phillips, Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish, Proc Natl Acad Sci U S A 102(19) (2005) 6919-24.

[49] Z. Xu, D. Parra, D. Gomez, I. Salinas, Y.A. Zhang, L.V. Jorgensen, R.D. Heinecke, K. Buchmann, S. LaPatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proceedings of the National Academy of Sciences of the United States of America 110(32) (2013) 13097-13102.
[50] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Gersdorff Jørgensen, S.E. LaPatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat Commun 7 (2016) 10728.
[51] Y.A. Zhang, I. Salinas, J. Li, D. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat Immunol 11(9) (2010) 827-35.
[52] B.A. Goven, D.L. Dawe, J.B. Gratzek, Protection of channel catfish, *Ictalurus-punctatus* Rafinesque, against *Ichthyophthirius-multifiliis* Fouquet by immunization, Journal of Fish Biology 17(3) (1980) 311-316.
[53] J.L. Maki, H.W. Dickerson, Systemic and cutaneous mucus antibody responses of channel catfish immunized against the protozoan parasite *Ichthyophthirius multifiliis*, Clin Diagn Lab Immunol 10(5) (2003) 876-81.
[54] D.H. Xu, P.H. Klesius, Protective effect of cutaneous antibody produced by channel catfish, *Ictalurus punctatus* (Rafinesque), immune to *Ichthyophthirius multifiliis* Fouquet on cohabited non-immune catfish, J Fish Dis 26(5) (2003) 287-91.
[55] T. Clark, H. Dickerson, R. Findly, Immune-response of channel catfish to ciliary antigens of *Ichthyophthirius-multifiliis*, Developmental and Comparative Immunology 12(3) (1988) 581-594.
[56] R.C. Findly, X.G. Zhao, J. Noe, A.C. Camus, H.W. Dickerson, B cell memory following infection and challenge of channel catfish with *Ichthyophthirius multifiliis*, Developmental and Comparative Immunology 39(3) (2013) 302-311.
[57] R. Hines, D. Spira, Ichthyophthiriasis in mirror carp *Cyprinus-carpio* (L). 5. Acquired immunity, Journal of Fish Biology 6(4) (1974) 373-&.
[58] T. Clark, H. Dickerson, J. Gratzek, R. Findly, *In vitro* response of *Ichthyophthirius-multifiliis* to sera from immune channel catfish, Journal of Fish Biology 31 (1987) 203-208.
[59] G. Houghton, R. Matthews, Immunosuppression in juvenile carp, *Cyprinus-carpio* L - The effects of the corticosteroids triamcinolone acetone and hydrocortisone 21 - hemisuccinate (cortisol) on acquired-immunity and the humoral antibody-response to *Ichthyophthirius-multifiliis* Fouquet, Journal of Fish Diseases 13(4) (1990) 269-280.
[60] O.N. Bauer, Immunity of fish occurring in infections with *Ichthyophthirius multifiliis* Fouquet, 1876, Doklady Novaia erviia 93 (1953) 377-79.
[61] K. Ling, Y. Sin, T. Lam, Protection of goldfish against some common ectoparasitic protozoans using *Ichthyophthirius multifiliis* and *Tetrahymena-pyriformis* for vaccination, Aquaculture 116(4) (1993) 303-314.
[62] M. Burkart, T. Clark, H. Dickerson, Immunisation of channel catfish, *Ictalurus-punctatus* Rafinesque, againt *Ichthyophthirius-multifiliis* (Fouquet) - killed versus live vaccine, Journal of Fish Diseases 13(5) (1990) 401-410.
[63] M. Dalgaard, K. Buchmann, A. Li, Immunization of rainbow trout fry with *Ichthyophthirius multifiliis* sonicate: protection of host and immunological changes, Bulletin of the European Association of Fish Pathologists 22(5) (2002) 288-297.
[64] D.H. Xu, P.H. Klesius, C.A. Shoemaker, Effect of immunization of channel catfish with inactivated trophonts on serum and cutaneous antibody titers and survival against *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 26(4) (2009) 614-618.

[65] X. Wang, T. Clark, J. Noe, H. Dickerson, Immunisation of channel catfish, *Ictalurus punctatus*, with *Ichthyophthirius multifiliis* immobilisation antigens elicits serotype-specific protection, Fish & Shellfish Immunology 13(5) (2002) 337-350.
[66] D. Xu, V. Panangala, V. van Santen, K. Dybvig, J. Abernathy, P. Klesius, Z. Liu, R. Russo, Molecular characteristics of an immobilization antigen gene of the fish-parasitic protozoan *Ichthyophthirius multifiliis* strain ARS-6, Aquaculture Research 40(16) (2009) 1884-1892.
[67] A. Leff, T. Yoshinaga, H. Dickerson, Cross immunity in channel catfish, *Ictalurus-punctatus* (Rafinesque), against 2 immobilization serotypes of *Ichthyophthirius multifiliis* (Fouquet). Journal of Fish Diseases 17(4) (1994) 429-432.
[68] A. Swennes, R. Findly, H. Dickerson, Cross-immunity and antibody responses to different immobilisation serotypes of *Ichthyophthirius multifiliis*, Fish & Shellfish Immunology 22(6) (2007) 589-597.
[69] Y. Lin, G. Cheng, X. Wang, T. Clark, The use of synthetic genes for the expression of ciliate proteins in heterologous systems, Gene 288(1-2) (2002) 85-94.
[70] J. Gaertig, Y. Gao, T. Tishgarten, T. Clark, H. Dickerson, Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*, Nature Biotechnology 17(5) (1999) 462-465.
[71] J. Jayaram, A. Papoyan, Y. Bisharyan, D. Cassidy-Hanley, X.J. Zhang, P. Colussi, J.A. Appleton, L. Gagliardo, T.G. Clark, An Alternative Platform for Rapid Production of Effective Subunit Vaccines, Biopharm International (2010) 6-13.
[72] Y. Bisharyan, Q. Chen, M. Hossani, A. Papoyan, T. Clark, Cadmium effects on *Ichthyophthirius*: evidence for metal-sequestration in fish tissues following administration of recombinant vaccines, Parasitology 126 (2003) S87-S93.
[73] M. Heidarieh, S. Moodi, K.K. Katuli, H. Unger, Biochemical effects of encapsulated radiovaccine via alginate nanoparticles as useful strategy for booster in immunized rainbow trout against *Ichthyophthirius multifiliis*, Acta Scientiae Veterinariae 43 (2015) 11.
[74] M. Heidarieh, A. Diallo, S. Moodi, V. Taghinejad, M. Akbari, A. Monfaredan, Gene expression analysis in rainbow trout (*Oncorhynchus mykiss*) skin: immunological responses to radiovaccine against *Ichthyophthirius multifiliis*, Revue De Medecine Veterinaire 166(7-8) (2015) 234-242.
[75] J.Y. Yao, X.M. Yuan, Y. Xu, W.L. Yin, L.Y. Lin, X.Y. Pan, G.L. Yang, C.F. Wang, J.Y. Shen, Live recombinant Lactococcus lactis vaccine expressing immobilization antigen (i-Ag) for protection against *Ichthyophthirius multifiliis* in goldfish, Fish & Shellfish Immunology 58 (2016) 302-308.
[76] L.v.G. Jorgensen, P.W. Kania, K.J. Rasmussen, A.H. Mattsson, J. Schmidt, A. Al-Jubury, A. Sander, A. Salanti, K. Buchmann, Rainbow trout (*Oncorhynchus mykiss*) immune response towards a recombinant vaccine targeting the parasitic ciliate *Ichthyophthirius multifiliis*, Journal of Fish Diseases (2017), DOI: 10.1111/jfd.12653
[77] OECD, OECD Guidelines for the Testing of Chemicals. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-2-effects-on-biotic-systems_20745761OECD Guidelines for the Testing of Chemicals. (Accessed 9.01.2017 2017).
[78] C.W. Stiles, Report on a parasitic protozoan observed on fish in aquarium, Bulletin of the United States Fish Commission, 1893, pp. 173-190.
[79] A.D. Butcher, Ichthyophthiriasis in Australian trout hatchery, The Progressive Fish-Culturist, 1947, pp. 21-26.
[80] D. Cross, Review of methods to control ichthyophthiriasis, Progressive Fish-Culturist 34(3) (1972) 165-&.

[81] L. Aihua, K. Buchmann, Temperature- and salinity-dependent development of a Nordic strain of *Ichthyophthirius multifiliis* from rainbow trout, Journal of Applied Ichthyology 17(6) (2001) 273-276.
[82] D.S. Miron, L.V.F. Silva, J.I. Golombieski, B. Baldisserotto, Efficacy of Different Salt (NaCl) Concentrations in the Treatment of *Ichthyophthirius multifiliis-Infected*Silver Catfish, *Rhamdia quelen*, Fingerlings, Journal of Applied Aquaculture, 2008, pp. 155-161.
[83] A. Maceda-Veiga, J. Cable, Efficacy of sea salt, metronidazole and formalin-malachite green baths in treating *Ichthyophthirius multifiliis* infections of mollies (*Poecilia sphenops*), Bulletin of the European Association of Fish Pathologists 34(5) (2014) 182-186.
[84] C. Mifsud, S.J. Rowland, Use of salt to control ichthyophthiriosis and prevent saprolegniosis in silver perch, *Bidyanus bidyanus*, Aquaculture Research 39(11) (2008) 1175-1180.
[85] F. Garcia, R.Y. Fujimoto, M.L. Martins, F.R. Moraes, Protozoan parasites of *Xiphophorus* spp. (Poeciliidae) and their relation with water characteristics, Arquivo Brasileiro De Medicina Veterinaria E Zootecnia 61(1) (2009) 156-162.
[86] L.D. Garcia, A.G. Becker, M.A. Cunha, B. Baldisserotto, C.E. Copatti, D. Kochhann, Effects of Water pH and Hardness on Infection of Silver Catfish, *Rhamdia quelen*, Fingerlings by *Ichthyophthirius multifiliis*, Journal of the World Aquaculture Society 42(3) (2011) 399-405.
[87] Z. Rychlicki, Eradication of *Ichthyophthirius multifiliis* in carp, Food and Agriculture Organization Fisheries Reports, 1968, pp. 361-364.
[88] E. Amlacher, Textbook of Fish Diseases, TFH publications, Neptune City, New Jersey, 1970.
[89] D. Tieman, A. Goodwin, Treatments for ich infestations in channel catfish evaluated under static and flow-through water conditions, North American Journal of Aquaculture 63(4) (2001) 293-299.
[90] D.H. Xu, Q.Z. Zhang, D. Zhang, Two in vitro methods for screening potential parasiticides against *Ichthyophthirius multifiliis* using *Tetrahymena thermophila*, Journal of Fish Diseases 39(3) (2016) 285-294.
[91] T. Wahli, M. Schmitt, W. Meier, Evaluation of alternatives to malachite green oxalate as therapeutant for ichthyophthiriosis in rainbow trout *Oncorhynchus-mykiss*, Journal of Applied Ichthyology-Zeitschrift Fur Angewandte Ichthyologie 9(3-4) (1993) 237-249.
[92] D. Alderman, Malachite green - a review, Journal of Fish Diseases 8(3) (1985) 289-298.
[93] S. Srivastava, R. Sinha, D. Roy, Toxicological effects of malachite green, Aquatic Toxicology 66(3) (2004) 319-329.
[94] D. Straus, B. Griffin, Prevention of an initial infestation of *Ichthyophthirius multifiliis* in channel catfish and blue tilapia by potassium permanganate treatment, North American Journal of Aquaculture 63(1) (2001) 11-16.
[95] J.M. Forwood, J.O. Harris, M. Landos, M.R. Deveney, Minimum effective concentrations of formalin and sodium percarbonate on the free-living stages of an Australian isolate of *Ichthyophthirius multifiliis*, Parasitology Research 113(9) (2014) 3251-3258.
[96] K. Buchmann, J. Bresciani, C. Jappe, Effects of formalin treatment on epithelial structure and mucous cell densities in rainbow trout, *Oncorhynchus mykiss* (Walbaum), skin, Journal of Fish Diseases 27(2) (2004) 99-104.
[97] C. Sommerville, R. Endris, T. Bell, K. Ogawa, K. Buchmann, D. Sweeney, World association for the advancement of veterinary parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides for fish, Veterinary Parasitology 219 (2016) 84-99.
[98] EU, EU directive 2004/37/EC. https://osha.europa.eu/da/legislation/directives/directive-2004-37-ec-carcinogens-or-mutagens-at-work.

[99] M.o. employment, Kræftbekendtgørelsen, 2015. https://www.retsinformation.dk/Forms/R0710.aspx?id=176604 - id63536669-60b9-4dca-80c1-10bb719f1ab8.
[100] J.N. de Melo, W. Carneseca, M.L. Martins, J.L.P. Mourio, *In vitro* and *in vivo* study of chlorine dioxide to treat *Ichthyophthirius multifiliis* in silver catfish *Rhamdia quelen*, Boletim Do Instituto De Pesca 41(4) (2015) 987-993.
[101] H. Ogut, N. Gunduz, R. Colak, M. Aslan, Using Fibonacci dose escalation to determine the minimum effective concentrations of copper sulphate and chloramine-T for eradication of *Ichthyophthirius multifiliis*, Fresenius Environmental Bulletin 24(12B) (2015) 4650-4657.
[102] F.J. Sutili, L.T. Gressler, A.C. Vargas, C.C. Zeppenfeld, B. Baldisserotto, M.A. Cunha, The use of nitazoxanide against the pathogens *Ichthyophthirius multifiliis* and *Aeromonas hydrophila* in silver catfish (*Rhamdia quelen*), Veterinary Parasitology 197(3-4) (2013) 522-526.
[103] L. Pedersen, N.H. Henriksen, Semi-continuously addition of peracetic acid to a flow-through fish farm, Journal of Cleaner Production 142 (2017).
[104] M. Bruzio, K. Buchmann, The effect of per acetic acid products on parasites causing white spot disease, Fish farmer 33 (2010) 25-27.
[105] K. Buchmann, P. Jensen, K. Kruse, Effects of sodium percarbonate and garlic extract on *Ichthyophthirius multifiliis* theronts and tomocysts: *In vitro* experiments, North American Journal of Aquaculture 65(1) (2003) 21-24.
[106] E. Union, DIRECTIVE 2004/28/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL, 2004. http://www.biosafety.be/PDF/2004_28.pdf. (Accessed 07.04.2017 2017).
[107] R. Jaafar, K. Buchmann, Toltrazuril (Baycox (R) vet.) in feed can reduce *Ichthyophthirius multifiliis* invasion of rainbow trout (Salmonidae), Acta Ichthyologica Et Piscatoria 41(1) (2011) 63-66.
[108] G.M. Valladão, S.U. Gallani, C.V. Ikefuti, C. da Cruz, N. Levy-Pereira, M.V. Rodrigues, F. Pilarski, Essential oils to control ichthyophthiriasis in pacu, *Piaractus mesopotamicus* (Holmberg): special emphasis on treatment with *Melaleuca alternifolia*, Journal of Fish Diseases 39(10) (2016) 1143-52.
[109] D. Lin, Y. Hua, Q. Zhang, D. Xu, Y. Fu, Y. Liu, S. Zhou, Evaluation of medicated feeds with antiparasitical and immune-enhanced Chinese herbal medicines against *Ichthyophthirius multifiliis* in grass carp (*Ctenopharyngodon idellus*), Parasitology Research 115(6) (2016) 2473-2483.
[110] Y. Fu, Q. Zhang, D. Xu, B. Wang, J. Liang, D. Lin, Cynatratoside-C efficacy against theronts of *Ichthyophthirius multifiliis*, and toxicity tests on grass carp and mammal blood cells, Diseases of Aquatic Organisms 117(1) (2015) 13-20.
[111] W. Zheng, C. Yan, Y. Zhang, Z. Li, Z. Li, X. Li, Z. Wang, X. Wang, W. Chen, X. Yu, Antiparasitic efficacy of Gracillin and Zingibernsis newsaponin from Costus speciosus (Koen ex. Retz) Sm. against *Ichthyophthirius multifiliis*, Parasitology 142(3) (2015) 473-479.
[112] Y. Fu, Q. Zhang, D. Xu, H. Xia, X. Cai, B. Wang, J. Liang, Parasiticidal effects of *Morus alba* root bark extracts against *Ichthyophthirius multifiliis* infecting grass carp, Diseases of Aquatic Organisms 108(2) (2014) 129-136.
[113] J. Liang, Y. Fu, Q. Zhang, D. Xu, B. Wang, D. Lin, Identification and Effect of Two Flavonoids from Root Bark of Morus alba against *Ichthyophthirius multifiliis* in Grass Carp, Journal of Agricultural and Food Chemistry 63(5) (2015) 1452-1459.
[114] X. Shan, Q. Meng, Y. Kang, Y. Bian, Y. Gao, W. Wang, A. Qian, Isolation of active compounds from methanol extracts of *Toddalia asiatica* against *Ichthyophthirius multifiliis* in goldfish (*Carassius auratus*), Veterinary Parasitology 199(3-4) (2014) 250-254.

[115] Q. Zhang, D. Xu, P. Klesius, Evaluation of an antiparasitic compound extracted from *Galla chinensis* against fish parasite *Ichthyophthirius multifiliis*, Veterinary Parasitology 198(1-2) (2013) 45-53.
[116] K. Song, F. Ling, A. Huang, W. Dong, G. Liu, C. Jiang, Q. Zhang, G. Wang, *In vitro* and *in vivo* assessment of the effect of antiprotozoal compounds isolated from *Psoralea corylifolia* against *Ichthyophthirius multifiliis* in fish, International Journal For Parasitology-Drugs and Drug Resistance 5(2) (2015) 58-64.
[117] F. Ling, C. Lu, X. Tu, Y.L. Yi, A.G. Huang, Q.Z. Zhang, G.X. Wang, Antiprotozoal screening of traditional medicinal plants: evaluation of crude extract of *Psoralea corylifolia* against *Ichthyophthirius multifiliis* in goldfish, Parasitology Research 112(6) (2013) 2331-2340.
[118] Y. Gao, X. Shan, Y. Kang, Y. Sheng, L. Chen, W. Wang, H. Ma, Evaluation of an anti-parasitic compound extracted from *Streptomyces* sp HL-2-14 against fish parasite *Ichthyophthirius multifiliis*, Parasitology 142(7) (2015) 910-916.
[119] J. Yao, Y. Xu, W. Yin, X. Yuan, L. Lin, T. Xu, M. Zuo, X. Pan, J. Shen, Evaluation of nystatin isolated from *Streptomyces griseus* SDX-4 against the ciliate, *Ichthyophthirius multifiliis*, Parasitology Research 114(4) (2015) 1425-1431.
[120] S. Picon-Camacho, E. Leclercq, J. Bron, A. Shinn, The potential utility of the leopard pleco (*Glyptoperichthys gibbiceps*) as a biological control of the ciliate protozoan *Ichthyophthirius multifiliis*, Pest Management Science 68(4) (2012) 557-563.
[121] K. Buchmann, Epidemiology of pseudodactylogyrosis in an intensive eel-culture system, Diseases of Aquatic Organisms 5(2) (1988) 81-85.