Transmission and control of Fish-borne Zoonotic Trematodes in aquaculture

Annette S. Boerlage

Thesis committee

Promotors
Prof. dr. ir. M.C.M. de Jong
Professor of Quantitative Veterinary Epidemiology
Wageningen University

Prof. dr. J.A.J. Verreth
Professor of Aquaculture and Fisheries
Wageningen University

Co-promotor
Dr. ir. E.A.M. Graat
Assistant professor, Quantitative Veterinary Epidemiology Group
Wageningen University

Other members
Prof. dr. ir. I.J.M. de Boer, Wageningen University
Prof. dr. I.A. Gardner, University of Prince Edward Island, Charlottetown, Canada
Prof. dr. ir. W. Takken, Wageningen University
Dr. M.E.E. Kretzschmar, University Medical Center (UMC) Utrecht and National Institute for Public Health and the Environment (RIVM), Bilthoven

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Annette S. Boerlage

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Annette S. Boerlage

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ABSTRACT

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Fish-borne Zoonotic Trematodes (FZTs) affect the health of millions of humans worldwide. For persistence, the life cycle of FZTs depends on aquatic snails, fish, and definitive hosts like humans, pigs or chickens. Definitive hosts can become infected by eating raw or undercooked fish. Integrated Agriculture Aquaculture (IAA) systems improve the livelihood of small scale farmers, but may enhance transmission of FZTs because all types of hosts and all transmission routes can be present on a single farm. This thesis combines experiments, statistical analyses and mathematical modelling to gain insight into transmission mechanisms of FZTs to fish in aquaculture and to use this insight to compare and discuss control measures against FZTs. Currently, medication of humans is the main strategy to control FZTs. Modelling indicated that this does not lead to elimination of FZTs because both humans and definitive hosts other than humans will maintain the life cycle of FZTs independently. Treatment of (a part of) these host types may eliminate FZTs, e.g. treating all humans and 54% of definitive hosts other than humans. Aquaculture may provide opportunities for control of FZTs by adapting management measures. Experiments showed that smaller fish get more often and more heavily infected with FZTs than larger fish; common carp (*Cyprinus carpio*) of more than 50 g rarely acquire new infections. Once carp are infected, FZTs persist for at least 27 weeks, implying that harvestable fish still contain FZTs and, therefore, are a risk to human health. In most IAA systems, fish are kept FZT free until 0.5 g before being stocked into a fish pond where they are very likely to be exposed to FZTs. Stocking fish at more than 25 g, or at more than 14 g in combination with treating all humans with anthelmintics, may lead to elimination of FZTs. Also, control of snails by either decreasing density or increasing mortality of snails may lead to elimination of FZTs in aquaculture. Farmers and policy makers should evaluate which combination of control measures is attractive to them.

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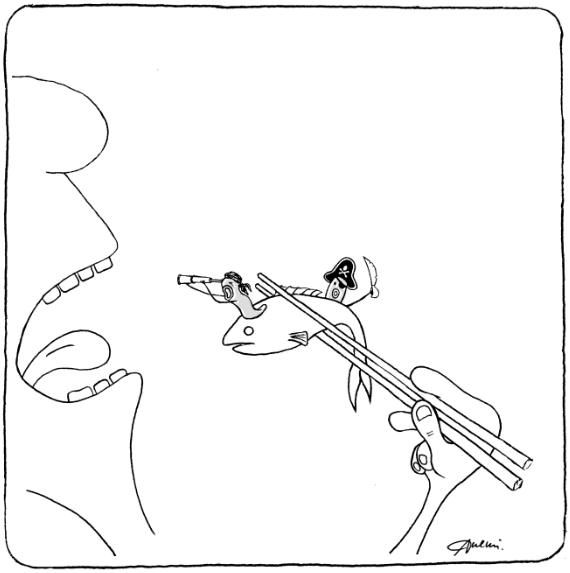
1

General introduction



Introduction

More than 10% of the human population is at risk of infection with crustacea-, plant-, or fish-borne zoonotic trematodes. These trematodes affect the health of more than 40 million people (Abdussalam et al., 1995; WHO, 2011) and affect economics in terms of loss of productivity, absenteeism, and health care costs. Furthermore, food safety issues can affect trade (Roberts et al., 1994; WHO, 1995, 2004).

This thesis focuses on fish-borne zoonotic trematodes (FZTs). Humans can become infected with FZTs when they consume raw or undercooked fish. The association between human infections and FZTs in fish is well described for fisheries, but has been neglected for aquaculture until about 15 years ago (Lima dos Santos and Howgate, 2011). This is surprising, because 40% of the worldwide production of fish origins from aquaculture (FAO, 2012). Farmers can manage fish in aquaculture, which may provide opportunities for control of FZTs. This thesis combines experiments, statistical analysis and mathematical modelling of FZT in aquaculture to improve understanding of the impact of several control measures. This first chapter is an introduction on FZTs in general, FZTs in Vietnam, FZTs in aquaculture, control measures against FZTs in humans and control measures against FZTs on aquaculture farms. It ends with the objective and outline of this thesis.

General

The life cycle of FZTs depends on three types of hosts; primary snail hosts, secondary fish hosts, and definitive hosts like humans, dogs or fish-eating birds (Fig. 1).

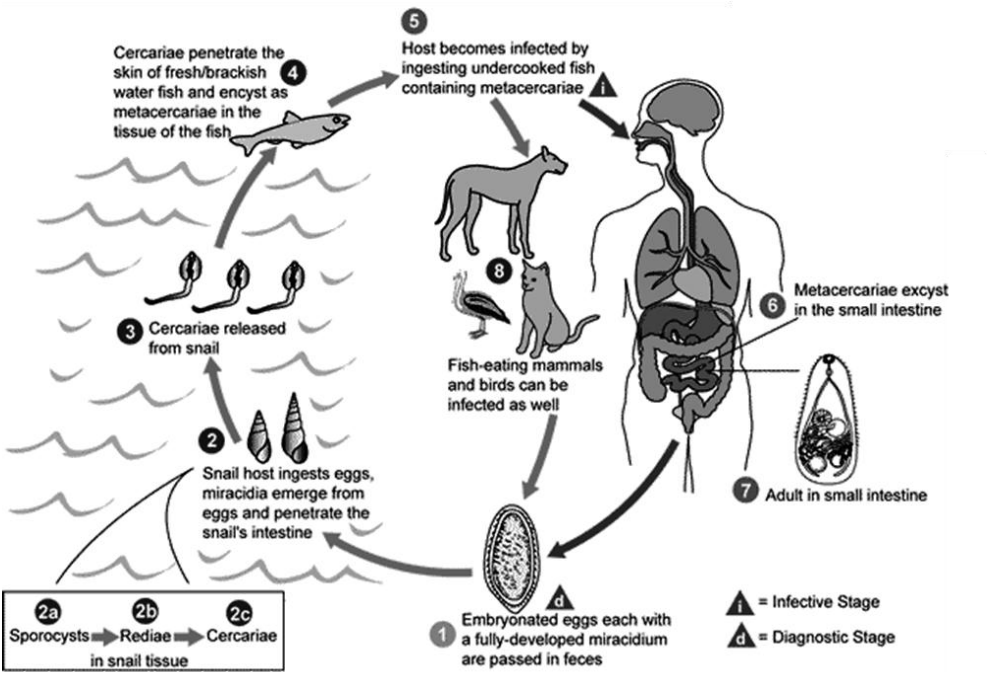


Figure 1. The life cycle of FZTs (CDC, 2012).

Definitive hosts contain the adult worm stage of FZTs that excrete eggs. Faeces with eggs are excreted into the environment (Fig. 1, bullet 1), where eggs are eaten by aquatic snails (Smith, 1984; Chen et al., 1994; King and Scholz, 2001; Kaewkes, 2003; Lun et al., 2005; Dung et al., 2010). In about 80 days (reviewed by Chen et al., 1994), eggs in snails develop into miracidiae, sporocysts, rediae and eventually cercariae (bullet 2). Snails shed cercariae into the water (bullet 3). Cercariae live about 3 days (Lo and Lee, 1996b) during which they search for fish. They penetrate into the muscles of fish and develop into metacercariae (bullet 4). After definitive hosts consume raw or almost raw infected fish (bullet 5 and 8), metacercariae excyst and develop into adult worms (bullet 6). Worms can survive 15 to 25 years in humans (Chen et al., 1994). FZTs are categorized into liver and intestinal flukes based on the target organs of worms (bullet 7).

All fish-borne zoonotic liver flukes belong to the family Opisthorchiidae. The best-known species, *Clonorchis sinensis,* *Opisthorchis viverrini* and *Opisthorchis felineus,* are endemic in Asia and Eastern Europe (Rim, 1982a, b). About 17 million people are infected with these species, and another 67 million people are at risk (Rim, 1982b; WHO, 1995; Keiser and Utzinger, 2005; Sithithaworn et al., 2008). Most infections are mild and asymptotic. Heavy and/or chronic infections can lead to damage of bile ducts and liver epithelium, which may lead to cholangitis, choledocholithiasis, pancreatitis and cholangiocarcinoma (Hamilton and Aaltonen, 2000; Watanapa and Watanapa, 2002; Sithithaworn and Haswell-Elkins, 2003; Chai et al., 2005; Rim, 2005). The International Agency for Research on Cancer considers *C. sinensis, O. viverrini* and *O. felineus* carcinogenic to humans for their association with cholangiocarcinoma (Vatanasapt et al., 1995; WHO, 2012). Early detection of cholangiocarcinoma is difficult and prognosis is not favorable; the majority of patients that are diagnosed with cholangiocarcinoma in a hospital survive less than 12 months (Anderson et al., 2004).

Fish-borne zoonotic intestinal flukes belong to the families Echinostomatidae, Heterophyidae, or Nanophyetidae. They occur in the tropics and sub-tropics around the globe (e.g. Martin, 1958; Morgan and Blair, 1998; Scholz et al., 2001; Fried et al., 2004; Umadevi and Madhavi, 2006; Detwiler et al., 2010; Sripa et al., 2010). So far, estimates on the number of infected people or population at risk worldwide are not available. Echinostomatidae and Heterophyidae are mainly parasites of birds and mammals. At least 11 Echinostomatidae species and 35 Heterophyidae species infect humans. Among them are *Echinostoma ilocanum, E. revolutum, Heterophyes heterophyes, Haplorchis pumilio*, *Centrocestus formosanus* and *Metagonimus yokogawai* (Yu and Mott, 1994; Chai et al., 2005; Sripa et al., 2010). Human infections with intestinal flukes are in general asymptomatic or accompanied by mild intestinal discomfort, like inflammations and ulceration of the mucosa and superficial necrosis. Heavy and/or chronic infections can lead to diarrhea, mucus-rich stool, pain, anorexia, nausea and vomiting. In very rare cases, minute eggs produced by adult worms are trapped in smaller vessels and capillaries of vital organs, like spinal cord, heart and brain, where egg emboli can cause death (Velasquez, 1982; reviewed by Sripa et al., 2010). Intestinal flukes can be misdiagnosed as liver flukes (Ditrich et al., 1992a).

In the family Nanophyetidae, the zoonotic species *Nanophyetus salmincola* is associated with salmon, but can also occur in non-salmonid fish. It is endemic in the Pacific Northwest of Canada and United States (Booth et al., 1984; Headley et al., 2011). Clinical symptoms in humans are similar to those reported for Echinostomatidae and Heterophyidae (Yu and Mott, 1994). *N. salmincola* is known for being a vector for the bacteria *Neorickettsia helminthoeca* that can cause fatal infections in dogs, foxes and coyotes. *N. helminthoeca* has not been associated with human disease (Yu and Mott, 1994).

FZTs in Vietnam

FZTs occur worldwide. Information on population at risk and infected population per country can be found in e.g. WHO (1995; 2011), Chai et al. (2005) and Keiser and Utzinger (2005). FZTs in Vietnam are used as example in this thesis, therefore a short history and overview on FZTs in Vietnam follows.

The first report on FZTs in Vietnam dates from 1887. Grall observed that 73% of red river delta inhabitants living in and around Haiphong and Hanoi were infected with *C. sinensis* (reviewed by Komiya, 1966). Until now, *C. sinensis* has only been reported in Northern provinces of Vietnam (Kino et al., 1998; reviewed by De et al., 2003; Nontasut et al., 2003; Verle et al., 2003; Dung et al., 2007). The highest percentage infected hosts was 26% of 1155 humans (Thi Cam et al., 2008), 13.3% of 173 snails, 56.4% of 101 small fish, all 25 large fish (Kino et al., 1998), 92% of 26 cats and 67% of 12 dogs (reviewed by De et al., 2003). A more recent study indicated a much lower prevalence of *C. sinensis* in fish in the same area, 1.5% of 797 fish were infected (Phan et al., 2010b).

O. felineus was first reported in Vietnam in 1907 (reviewed by De et al., 2003). Later, it was concluded that the observed trematodes might have been the species *O. viverrini*, because it was the only report of *O. felineus* and they have not been reported elsewhere in Southeast Asia (WHO, 1995). *O. viverrini* occurs in the Middle and Southern part of Vietnam. The highest percentage infected hosts was 37% of 670 humans, 29% of 70 fish, 8% of 964 snails, 60% of 10 dogs and 33% of 6 cats (reviewed by De et al., 2003).

Overall, the liver flukes are endemic in 32 out of the 63 provinces in Vietnam (WHO, 2011). The estimated Vietnamese population at risk is 10 million people for both *C. sinensis* and *O. viverrini* (J.Y. Chai pers. comm. in Keiser and Utzinger, 2005).

Heterophyid infections in humans in Vietnam were reported for the first time in 2007. *Haplorchis pumilio* was found in 65% of 615 humans, *H. taichui* in 45%, and *H. yokogawai* in 2% (Dung et al., 2007). This relative late report of the occurrence of intestinal flukes in this area is remarkable, because intestinal flukes were commonly encountered in countries that surround Vietnam (Ditrich et al., 1992a; WHO, 1995; De et al., 2003). Misidentification of eggs may be the reason that Heterophyidae were not reported earlier (Dung et al., 2007). Intestinal flukes were found in 13% of 6367 snails (Dung et al., 2010). In fish, Heterophyidae were reported in 41 freshwater and 3 marine fish species with prevalences up to 100% (Arthur and Te, 2006; Hop et al., 2007; Thien et al., 2007; Thu et al., 2007; Chi et al., 2008; Vo et al., 2008; Chi et al., 2009; Thien et al., 2009; Skov et al., 2009; Phan et al., 2010a; 2010b; 2010c). In definitive hosts other than humans, 70% of 20 cats, 57% of 25 dogs (Lan Anh et al., 2009a), 14% of 114 pigs (Lan Anh et al., 2009b), 12% of 50 chickens and 30% of 50 ducks (Anh et al., 2010a) were infected with intestinal flukes.

FZTs in aquaculture

Aquaculture is an increasingly important production sector for high value protein worldwide that produced 59.9 million tons of products in 2010. Asia produced 89% of the total, the top three producing countries being China with 36.7 million ton, India with 4.6 million ton, and Vietnam with 2.7 million ton (FAO, 2012). Asia is also the continent where FZTs occur most and where FZTs have been reported in aquaculture (Keiser and Utzinger, 2005; Lima dos Santos and Howgate, 2011; WHO, 2011).

Most aquaculture practices in Asia use manure from livestock and possibly human faeces, because the nutrients in manure nourish the natural food web in a fishpond (Goddard, 1996; Little and Edwards, 2003). Extensive aquaculture systems can rely solely on input of manure, whereas more intensive aquaculture systems replace manure by commercial fish feed to obtain higher yields (Baluyut, 1989; Goddard, 1996). Manure may contain FZT eggs, implying that the use of manure in aquaculture can enhance transmission from infected definitive hosts to snails (Sapkota et al., 2008; Dung et al., 2010). Indeed, in aquaculture systems that rely on commercial feed, a lower percentage of fish are infected with FZTs than in systems where manure is used as pond fertilizer (Thien et al., 2009).

Integrated Agriculture-Aquaculture (IAA) farming is a common aquaculture practice in Asia (FAO, 2001). This type of farming is economically and ecologically sustainable, because the wastes of fish ponds, crops (mainly rice), vegetables, and livestock (mainly manure of pigs, chickens or ducks) are recycled within the farm (Baluyut, 1989; Prein, 2002; Little and Edwards, 2003). A disadvantage of this system is that all hosts and contact possibilities necessary to maintain the life cycle of FZTs can be present on a single farm (Naegel, 1990). Manure is used as pond fertilizer, both snails and fish inhabit the pond which leads to transmission from snail to fish, farmers may have traditional habits of eating raw fish (Phan et al., 2011) and farmers may feed kitchen waste to their livestock, leading to transmission from fish to definitive hosts. IAA farms are used as example in this thesis.

Control and prevention of FZTs in humans

Most countries where FZTs are endemic aim at reducing morbidity in humans (Prichard et al., 2012). The core control measure against FZTs is chemotherapeutic treatment of humans (WHO, 2011). Praziquantel is the only drug used against FZTs and has cure rates of 100% in humans (Dung et al., 2007; Keiser et al., 2010; Soukhathammavong et al., 2011; Waikagul et al., 2005; WHO, 2011). Chemotherapeutic treatment can be used targeted, e.g. to schoolchildren only, universal, i.e. to the whole community, or selectively, i.e. targeting the part of the population that is diagnosed as infected only (O'Lorcain and Holland, 2000). The World Health Organization (WHO) advises to complement chemotherapeutic treatment with public health interventions like educating humans about the risks of eating raw fish dishes (WHO, 2011). The latter has not been very successful, because traditional habits are not changed easily (Guoqing et al., 2001; WHO, 2004; 2011) and humans know that effective drugs are available (Phan et al., 2011). Another public health intervention is the treatment of aquaculture products. Products can become FZT-free by heating and cooling (Hamed and Elias, 1970; Fan, 1998; Cho et al., 2002; Zhang et al., 2003), salting, fermenting, marinating (Abdussalam et al., 1995; Sukontason et al., 1998; Beldsoe and Oria, 2001; Cho et al., 2002; Butt et al., 2004) and irradiation (Lee et al., 1989; Loaharanu and Murrell, 1994).

Control and prevention of FZTs on IAA farms

Humans regulate the production of fish in aquaculture, which provides opportunities for control of FZTs. Control in farm management can affect the life cycle of FZTs at different spots.

Farmers can prevent and cure infection of FZTs in non-human definitive hosts. Caged livestock, e.g., pigs, can be fed a fish-free diet (Lan Anh et al., 2009a). Free roaming livestock, pets and wild vertebrates, can be prevented from feeding on fish in the pond by fencing off the pond (Khamboonruang et al., 1997). Infected non-human definitive hosts can be cured by a treatment with praziquantel (Anh et al., 2010b).

Farmers can reduce transmission to snails if they stop the use of manure as pond fertilizer. This can be enforced by law, or at own initiative e.g. supported by education provided by a third party (WHO, 1995). Drains that lead manure from pens of caged livestock and human toilets can be redirected, e.g. to a sewage system (WHO, 2011). Alternatively, manure can be made FZT-free by heating, drying or treating manure with chemicals (Jimenez-Cisneros and Maya-Rendon, 2007). Free roaming definitive hosts, e.g. dogs, drop faeces around a pond. These faeces may be flushed into the pond during rain, which can be prevented by a cement barrier along the banks of the pond (Clausen et al., 2012b).

Farmers can control the density of snails in a pond by removing their breeding sites (i.e. vegetation on banks in the pond), manipulating the level and salinity of the water, flushing the pond, and removing the mud layer on the bottom of the pond that contains snails (Hoffman, 1970; Naegel, 1990; Khamboonruang et al., 1997; Venable et al., 2000; Clausen et al., 2012b). Furthermore, a net over the water inlet pipe may reduce immigration of snails into the pond (Clausen et al., 2012b). A chemical method to control the snail population is the administration of molluscicides to the pond (Hoffman, 1970). Biological methods are the introduction of predators of snails (e.g., ducks or black carps), competitive snail species, or snail diseases (Hoffman, 1970; Slootweg et al., 1992; Hansen and Perry, 1994; Madsen, 1990; Venable et al., 2000; Ben-Ami and Heller, 2001). Total eradication of snails from a pond may not be feasible (Slootweg et al., 1994), but that may not be necessary (see Smith et al., 2012).

Farmers can reduce the density of infected fish by curing infected fish with praziquantel (Van et al., 2009). However, this measure requires more research before it can be applied because there is no knowledge on the eco-toxicological impact of feeding praziquantel to fish. Import of FZT-infected fish on a farm can be prevented if farmers buy FZT-free fry or fingerlings only (Khamboonruang et al., 1997). There may be more opportunities for control of FZTs in aquaculture management, but the development of such measures requires more knowledge on transmission mechanisms to fish.

Sociocultural and economical aspects of control measures may determine if a control measure will be used by farmers. For example, the use of manure to fertilize a pond and the feeding of kitchen waste to livestock are among the characteristics that make IAA systems sustainable (Little and Edwards, 2003). Stopping those activities requires a fundamental change in attitude and traditional farming practices that may not be easily adopted and might be economically difficult to realize for the traditional small scale village farmer.

Objective of thesis

FZTs have a large impact on human health. Farmers manage fish in aquaculture, which may provide opportunities for control of FZTs in fish. Thus far, there are not many control measures describing control by changing the management of aquaculture. Development of such measures requires a better understanding of transmission mechanisms to fish. Furthermore, different control measures target different parts of the life cycle of FZTs and may also be used in combination. A better understanding of how control measures affect transmission dynamics is needed in order to predict which (combination of) control measures may be effective and which may not.

Therefore, the objective of this thesis is:

To obtain more insight in transmission mechanisms of FZTs in aquaculture by developing a mathematical model that is used as a tool to interpret observed epidemiological parameters, guide collection of reliable quantitative data in experiments and field studies and might lead to further understanding of transmission of FZTs in aquaculture. The model is used to compare and discuss control measures with the ultimate goal to enable science based recommendations and prioritizations for control measures.

Outline of thesis

Mathematical modelling is a powerful tool that is used to study infectious diseases since 1760 (Bernoulli reviewed by Anderson and May, 1990). For this thesis, at first a mathematical model is built that describes transmission of FZTs on an IAA farm. IAA farms in Nam Dinh province, Vietnam, are used as an example. On those farms, all types of hosts are infected with FZTs (Dung et al., 2007; Lan Anh et al., 2009a; 2009b; Dung et al., 2010; Phan et al., 2010b; 2011), implying that FZTs maintain themselves on the farms. The process of building a model provides insights into the population dynamics (De Jong, 1995). The insights are used to develop research questions and to design studies to improve our understanding of transmission of FZTs in aquaculture. Transmission of FZTs in aquaculture between snails and fish starts with excretion of cercariae by snails (Chapter 2), after which free-living cercariae find a fish and encyst in fish (Chapter 3–6).

In lakes and rivers, the spatial distribution of definitive hosts causes patchiness in trematodes infections in snail populations (e.g. Krist et al., 2000). In ponds however, the risk of type of land use bordering a sample site on FZT infections in snails sampled on that site is unknown. Chapter 2 describes an observational study that describes the distribution of trematodes in in snails in ponds at IAA farms in Nam Dinh province, Vietnam.

One of the factors not always consistent in the increasing number of observational studies (e.g. Chai et al,. 2005; Keiser and Utzinger, 2005; Lima dos Santos and Howgate, 2011) is the relation between fish weight and infection probability and/or attack rates in FZTs. Chapter 3 and 4 describe experiments to investigate the effect of fish size on transmission and gives implications for intervention.

Common carp are one of the most preferred freshwater fish species by consumers in Asia, the region where fish-borne zoonotic trematodes (FZTs) like Heterophyidae are most prevalent (Keiser and Utzinger, 2005; Mohan Day et al., 2005). At harvesting of fish, the percentage infected fish and FZT burden in fish depend on previous gain and subsequent survival of FZTs. Gain is investigated in Chapter 3 and 4, survival rates of FZTs in common carp are unknown. Chapter 5 describes an experiment to quantify the survival of FZT infections in common carp.

Transmission of FZTs to fish in aquaculture takes place in a more-or-less closed designated area where the dimensions of the water depth and surface area of the base of the fish enclosure can be regulated, as well as the number of fish. A different number of fish per designated area may have an effect on the number of trematodes per fish, which may be relevant to human health. It is therefore important to know whether transmission occurs in a density-dependent fashion. Chapter 6 describes an experiment to investigate the effect of density of cercariae and fish on transmission.

The results of Chapter 2 – 6 are used to improve the model. Chapter 7 describes this model and how it is used to compare and discuss control measures. The effect of chemotherapeutic treatment of humans, snail control and delayed stocking of fish on dynamics of FZT infection in hosts is calculated using the reproduction ratio (R), i.e. the average number of new cases caused by one typical infectious individual in a fully susceptible population (Diekmann et al., 1990).

Chapter 8, the General Discussion, starts with a discussion on transmission mechanisms and control in aquaculture. It continues with a section on practical implications for control on IAA farms, integrated control of FZTs and other pathogens and the methods used in this thesis. This chapter ends with options for future research and a summary of the main conclusions.

2

Distribution of trematodes in snails in ponds at integrated small-scale aquaculture farms

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

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Abstract

In integrated small-scale aquaculture farming, animal and human excreta maybe used as fish feed and pond fertilizer, thereby enhancing transmission of fish-borne zoonotic trematodes (FZTs) from final hosts, like humans, pigs and chickens, to snails. Areas within a pond could vary in trematode egg-load due to the immediate bordering land, and this might provide implications for control of these trematodes or sampling in field studies measuring FZT prevalence in snails. We therefore estimated the effect of bordering land use on prevalence and FZT burden in snails in different areas within small-scale aquaculture ponds. Nine sampling areas within a pond were assigned in six ponds. For each sampling area, about 120 *Melanoides tuberculata* snails were collected. Based on land use bordering a sampling area, these were categorized in 5 risk-categories: low-risk (road, rice planted in pond, agriculture, or middle of pond), human access point to pond, livestock sty (pigs or poultry), both human access point and livestock sty, and water connection to canal. In total, 5392 snails were collected. Percentages of snails with parapleurolophocercous cercariae varied between 6% in areas categorized as low-risk and areas with livestock sty only to 15% in areas with both human access point and livestock sty; only this 15% was significantly different from the prevalence in the low-risk category. Percentages of snails with xiphidio cercariae did not differ between risk-categories and varied between 5% and 10%. Mean snail size was 15.2 mm, and was significantly associated with both the probability of infection as well as parasite burden. Very small differences in parasite burden were found at different land use areas; the maximum difference was about 11 cercariae. This study demonstrated only small differences between areas surrounding a pond on risk of snails to be infected with fish-borne trematodes within different pond areas. In field studies on FZTs in *M. tuberculata* snails in ponds, sampling from ponds can therefore be done without considering areas within ponds.

Keywords: *Melanoides tuberculata,* integrated small-scale aquaculture ponds, risk assessment, trematode

Introduction

Fish-borne zoonotic trematodes (FZTs) affect more than 40 million people worldwide (WHO, 2004). Liver flukes (*Opisthorchiidae*) are known best because of their clinical importance (WHO, 2011). Intestinal flukes (e.g. *Heterophyidae*) cause less severe pathology in humans (Toledo et al., 2006), but impair food safety and quality, which has consequences for public health (Yu and Mott, 1994; Chai et al., 2005). The life-cycle of FZTs involves three types of hosts, primary intermediate snail host, secondary intermediate fish host, and final host like humans, pigs, cats or fish eating birds (Komiya, 1966; Sithithaworn et al., 2008). In this paper we focus on the role of snails. Asexual reproduction within the snail results in the release of cercariae over a period of months to years (Esch et al., 2002) and these cercariae might cause infection in fish which in turn are the reason for the food safety concern associated with FZTs.

In integrated small-scale aquaculture farming systems, fish ponds are combined with other agricultural activities within the boundaries of one farm (Prein, 2002). Besides much strength, a weakness of small-scale aquaculture farming systems is potential human health risk because of the use of animal and human excreta as pond fertilizers (Phan et al., 2011; Sapkota et al., 2008). These excreta may contain (antimicrobial-resistant) pathogenic bacteria (Petersen and Dalsgaard, 2003), viruses (Sapkota et al., 2008), and macro-parasites (Chai et al., 2005).

On small-scale aquaculture farms in Northern Vietnam (Dung et al., 2010), identical species of fish-borne trematodes were found in snails (Dung et al., 2010), fish (Phan et al., 2010b), humans (Dung et al., 2007) and other reservoir hosts (Anh et al., 2010a; Lan Anh et al., 2009a), suggesting the life-cycle of specific FZTs is maintained on these farms.

Spatial distribution of final hosts have been reported to cause patchiness in trematode infections in snail populations, especially when individual snail movement is low and snails get infected by ingesting trematode eggs (Esch and Fernandez, 1994; Hechinger and Lafferty, 2005; Krist et al., 2000; Jokela and Lively, 1995). Fish ponds in Northern Vietnam are typically surrounded by agriculture (crops and/or fruit trees), livestock sty for pigs or poultry with one or more outlets to the fish pond, farmers house with access point to the pond, duck sty with enclosure for ducks in the pond, water connection to a canal, road and rice planted in the pond (Dung et al., 2010; FAO, 2001). These different types of land use bordering a pond could lead to variation in contamination of different pond sections with trematode eggs and with that cause patchiness in FZT infection in snail populations within a pond. Dung et al. (2010) showed variation in prevalence of FZTs in snails among large canals, small canals, rice fields and small-scale aquaculture ponds, but so far information on within pond variation of fish-borne trematodes in snails is lacking. If certain types of land use would be risk factors, then there might be implications for sampling methodology in field studies involving snails and this might give insight in how to design control measures. Therefore, the objective of this study was to estimate the effect of bordering areas on prevalence and parasite burden of trematodes in snails within ponds on integrated small-scale aquaculture farms.

Materials and methods

Six ponds with high *Melanoides tuberculata* numbers (>250 snails scooped per person per hour, maximum sample time per pond 4 h) were sampled in September 2009. The ponds were located in Nghia Lac and Nghia Phu communes, Nam Dinh province, Vietnam. Both communes have a history of endemic FZTs and are known for raw-fish-eating behavior of humans and small-scale aquaculture farming (Dung et al., 2010; Phan et al., 2011). The six fish ponds ranged in size from 290 m^2 to 8190 m^2. Rice was only cultivated in pond 4 in about 25% of pond area. One pond was inhabited by ±400 ducks in an enclosure of ± 2330 m^2, no snails were found within the enclosure. Further description of ponds is shown in Table 1.

Sample sizes were calculated using WIN EPISCOPE 2.0 (Thrusfield et al., 2001) using 95% confidence (two tailed) and 80% power to detect a difference between 3 and 15% prevalence (Dung et al., 2010) between groups. This resulted in a sample size of 87 snails per group. As 20% of snails would not be alive (Dudgeon, 1986), at least 110 snails per group should be sampled. Based on this we have decided to sample 120 *M. tuberculata* snails per sampling area. Each pond was "divided" in 9 sampling areas, being the 4 corners, 4 sides between corners and the middle of the pond in rectangular ponds. For each of these areas, 1 or 2 types of land use bordering a sampling area were recorded (Table 1). Road, dike, rice cultured in pond, none (middle of the pond), or agriculture (crops and fruit trees) were considered as low-risk land use for infection of snails. Presence of livestock sty (pigs and/or poultry), human access point to pond or water connection to canal were considered as higher risk. Based on type of land use bordering a sampling area, they were categorized in 5 risk-categories: low-risk, human access point to pond, livestock sty, both human access point and livestock sty, and water connection to canal. In case two different types of land use (e.g. livestock sty and agriculture) bordered a sampling area, it was assigned to the risk-category with potentially the highest risk (in the example: livestock sty). It was assumed that both human access point and livestock sty was at highest risk, and was therefore categorized as a separate risk-category.

Table 1. Per pond, surface, number of sample areas, bordering areas, and reservoir hosts.

	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6
Pond surface (±m^2)	290	1580	750	8190	375	3775
No. of sample areas	8	9	9	9	9	9
No. of land use areas bordering a pond						
Agriculture	6	7	6	6	7	3
Livestock sty	-	1	2	1	-	6
Human access point	2	1	1	4	1	2
Water connection	-	1	-	1	1	1
Road	3	-	2	-	2	4
Dike	-	-	1	-	-	-
Rice	-	-	-	3	-	-
None (middle)	-	1	1	1	1	-
No. of reservoir hosts observed around pond						
Pig	-	-	3	-	-	-
Dog	2	1	2	1	1	4
Cat	2	-	1	1	2	1
Chicken	4	-	2	2	-	-
Duck	2	-	5	8	-	±400

From each sampling area within a pond, bottom sediment containing snails was filtered with a sieve. Processing of snail samples from 1 pond began in a laboratory about 1 h after sampling and was completed before continuing with the next pond. Snails were individually transferred to approximately 3 ml cercariae-free drinking water in glass tubes at approximately 24 ºC. After 24 h, shell height, and the number of cercariae shed was counted on a grit by stereomicroscope (for 65% of snails duplo counts were made by one person, and the two counts were averaged), and cercariae species were identified according to standard keys (Ditrich et al., 1992b; Schell, 1985; Scholz et al., 2000; Whitfield et al., 1975). Snails that did not shed cercariae were crushed and observed under stereomicroscope. If rediae or cercariae were present, this snail was recorded as false-negative.

Two prevalences were recorded: a snail was considered infected if (1) at least one cercariae was shed, (2) if cercariae were found after shedding or crushing. Cercariae burden was recorded after shedding only.

Statistical analyses were performed using SAS 9.2 (SAS Institute Inc., 2004). Infection with parapleurolophocercous or xiphidio cercariae in snails (yes/no) was analyzed with generalized linear models with a binomial distribution and a logit link function (i.e. logistic regression analysis) in which the variable land use, snail size and their interaction were included as explanatory variables. Linearity between logit and snail size was assessed as described by Dohoo et al. (2003). As snails within ponds and sampling areas nested within ponds cannot be regarded as independent observations, pond and area within pond were included as random effects using an exchangeable correlation structure. As area within pond was not significant, explained only 1.2% of non-explained variance for both cercariae types, and did not change *P*-values, it was excluded from the model. Results were expressed as prevalences and odds ratios.

An excess of zero counts (Fig. 1) leads to over-dispersion, which can be dealt with by using zero-inflation models. Such models split the process into a model for probability of a zero outcome by logistic regression and a model for counts using either a Poisson or a negative-binomial distribution (Dohoo et al., 2003). A zero-inflated model fitted better than a similar model without zero-inflation for parapleurolophocercous cercariae (Vuong test: $Z=3.01$, $P<0.01$), but not for xiphidio cercariae ($Z= -2.06$, $P=0.98$). Also, a negative-binomial distribution fitted better than a Poisson distribution (likelihood-ratio test $P<0.01$). Therefore, parapleurolophocercous and xiphidio cercariae burden were analyzed with zero-inflated generalized linear regression models with a negative-binomial distribution and a log link function. For both cercariae types the variables land use and snail size were included as explanatory variables. Dispersion parameters were respectively 2.2 and 3.5.

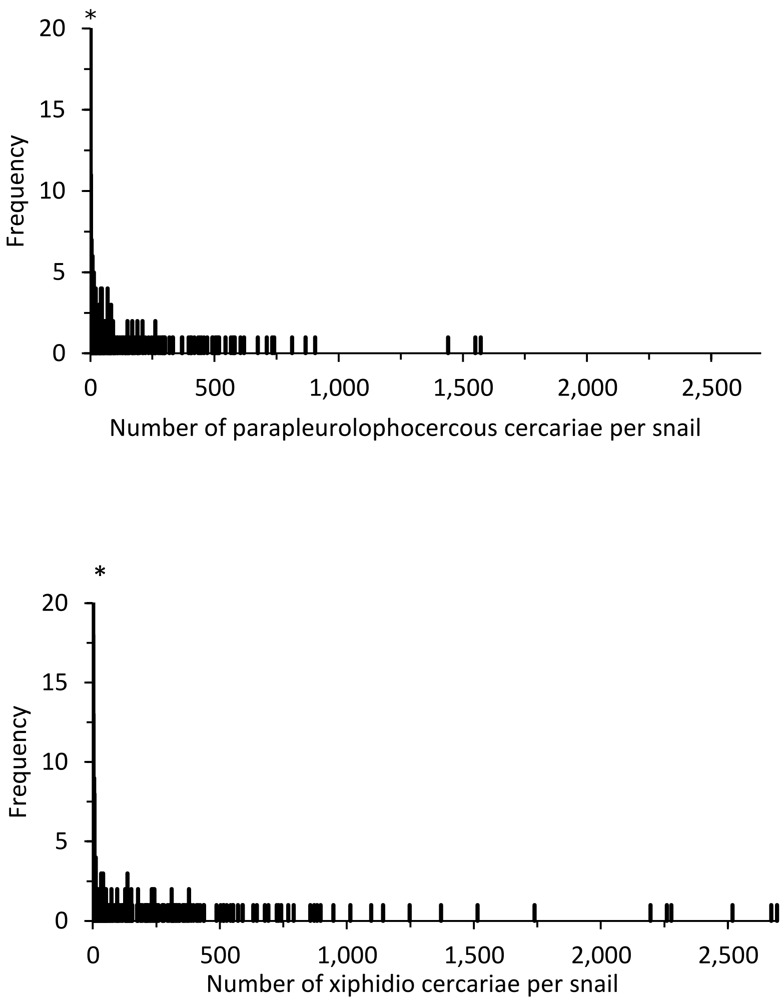

Figure 1. Frequency of snails per parapleurolophocercous and xiphidio cercariae burden. The y-axes were truncated: 4,854 snails did not shed cercariae.

Results

In total 5392 snails were collected (Table 2) and 13.8% of snails were infected. Xiphidio cercariae and parapleurolophocercous cercariae were observed in each pond, while the non-FZT *Transversotrema* sp. was found in 2 ponds. Three snails shed other types of cercariae (2 echinostome, 1 monostome), which were excluded from further study. After shedding and crushing, the prevalence of both parapleurolophocercous and xiphidio positive snails was 6.6%, of *Transversotrema* 0.6%. No mixed infections were found, each snail was infected with a single type of parasite. Crushing increased the observed prevalence of shedding, varying from no extra positive snails in one pond, to an increase of 4.7% in another pond.

Percentages of snails with parapleurolophocercous cercariae varied between 6% in areas categorized as low-risk and livestock sty only, to 14.5% in the area categorized as human access point and livestock sty at one site (Table 3). Overall, the variable land use was not significantly associated with the probability of snails to be infected (P=0.24). Only snails in the area with human access point and livestock sty at one site were significantly more often infected than snails in the low-risk area (OR=1.96, P<0.01). Percentage of non-explained variance by ponds was small, being 3.5%. Percentages of snails with xiphidio cercariae varied between 5.4% and 9.8%, and were not significantly different (P=0.30) between risk-categories of the variable land use (Table 3). Percentage of non-explained variance by ponds was 2.8%.

Snail size was normally distributed and on average 15.2 mm (SD=2.6; n=5392). For both parasite types, infected snails were on average larger than non-infected snails (P<0.01), and size of snails was significantly associated with the probability of snails to be infected (P<0.01) (Table 3). Per mm increase in snail size, the OR increased with 1.29 for parapleurolophocercous cercariae, implying that snails of e.g. 15 mm have a 3.52 increased odds of being infected compared to snails of 10 mm. For xiphidio cercariae, this was 1.18 per mm increase in snail size. There was no statistically significant interaction effect between the variables land use and snail size for both parasite types and deletion of either variable from the model did not change parameter estimate of the remaining variable. To visualize the effect of snail size, Fig. 2 shows the estimated percentage of infected snails with increasing snail size resulting from univariable analyses.

Table 2. Per pond and cercariae species, number of snails sampled, number and percentage of positive snails either by shedding only and by shedding and crushing, and median (min-max) cercariae for positive snails.

Pond	Cercaria species[a]	No. snails sampled per pond	No. (%) snails positive by shedding	No. (%) false-negative snails	No. (%) snails positive by shedding and crushing	Median (min-max) number cercariae[b]
1	Pleuro	801	3 (0.4)	0 (0)	3 (0.4)	8 (2-506)
	Xiphi	801	27 (3.4)	0 (0)	27 (3.4)	73 (1-588)[1]
2	Pleuro	1072	32 (3.0)	0 (0)	32 (3.0)	52 (2-812)
	Xiphi	1072	20 (1.9)	3 (0.3)	23 (2.2)	8 (1-946)
3	Pleuro	963	116 (12.1)	20 (2.1)	136 (14.1)	83 (1-709)[2]
	Xiphi	963	61 (6.3)	45 (4.7)	106 (11.0)	149 (1-1013)[3]
	Trans	963	0 (0)	1 (0.1)	1 (0.1)	-
4	Pleuro	1042	55 (5.3)	6 (0.6)	61 (5.9)	45 (1-1440)
	Xiphi	1042	33 (3.2)	11 (1.1)	44 (4.2)	187 (1-1514)
	Trans	1042	18 (1.7)	12 (1.2)	30 (2.9)	3 (1-10)
5	Pleuro	467	23 (4.9)	17 (3.6)	40 (8.6)	92 (3-905)
	Xiphi	467	18 (3.9)	19 (4.1)	37 (7.9)	165 (23-2670)
6	Pleuro	1047	49 (4.7)	35 (3.3)	84 (8.0)	69 (1-1571)[4]
	Xiphi	1047	83 (7.9)	37 (3.5)	120 (11.5)	127 (1-2693)[5]
Total	Pleuro	5392	278 (5.2)	78 (1.5)	356 (6.6)	69 (1-1571)[6]
	Xiphi	5392	242 (4.5)	115 (2.1)	357 (6.6)	126 (1-2693)[7]
	Trans	5392	18 (0.3)	13 (0.2)	31 (0.6)	3 (1-10)

[a] Pleuro: parapleurolophocercous cercariae; Xiphi: xiphidio cercariae; Trans: cercariae of *Transversotrema.*[b] Based on number of snails shedding cercariae unless indicated different by superscript. [1] n = 23; [2] n = 91; [3] n = 51; [4] n = 42; [5] n = 78; [6] n = 246; [7] n = 223. Differences are snails positive after shedding of which cercariae species was identified, but number cercariae not counted.

Table 3. Per cercariae species results of the multivariable model for infected snails (%) and cercariae burden (mean).

Cercariae species[a]	Variable	Risk-category	Frequency (no.)	Frequency (%)	Infected snails[c] Prev. (%)	Infected snails[c] OR (95% CI)	Infected snails[c] P_{Wald}[d]	Cercariae burden Frequency[e] (no.)	Cercariae burden Predicted mean (5-95 percentile)	Cercariae burden P_{Wald}[d]
Pleuro	Land use[b]	Low-risk	2959	54.9	6.0	Ref.		2928	6.9 (2.2-15.9)	Ref.
		Human access	910	16.9	7.0	1.35 (0.91-2.00)	0.14	905	8.3 (2.5-18.0)	0.03
		Livestock sty	926	17.2	5.8	0.70 (0.33-1.49)	0.36	917	6.0 (2.0-14.7)	0.39
		Human access and livestock sty	221	4.1	14.5	1.96 (1.51-2.54)	<0.01	215	17.4 (6.9-34.4)	<0.01
		Water connection	376	7.0	7.7	1.32 (0.86-2.02)	0.21	376	8.2 (3.0-16.5)	0.25
	Snail size (per mm)	Positive	*N*=356	avg=16.9 (±3.6)		1.29 (1.22-1.36)	<0.01	324		<0.01
		Negative	*N*=5036	avg=15.1 (±2.5)				5017		
Xiphi	Land use[b]	Low-risk	2959	54.9	5.7	Ref.		2928	9.1 (4.8-16.2)	Ref.
		Human access	910	16.9	6.7	1.22 (0.79-1.88)	0.37	905	14.0 (7.2-23.6)	<0.01
		Livestock sty	926	17.2	9.8	1.12 (0.35-3.58)	0.85	917	19.6 (11.0-34.6)	<0.01
		Human access and livestock sty	221	4.1	5.4	0.68 (0.34-1.36)	0.28	215	9.1 (5.2-14.9)	0.58
		Water connection	376	7.0	6.7	1.15 (0.58-2.27)	0.69	376	9.0 (5.1-14.7)	0.86
	Snail size (per mm)	Positive	*N*=357	avg=16.2 (±3.3)		1.18 (1.12-1.24)	<0.01	338		<0.01
		Negative	*N*=5035	avg=15.1 (±2.6)				5003		

[a] Pleuro: parapleurolophocercous cercariae; Xiphi: xiphidio cercariae. [b] Types of land use were assigned to risk-categories with potentially different risk of snails to be infected. Agriculture, road, rice and/or none were considered low-risk. Potentially high-risk categories were human access point to pond, livestock sty, both at the same waterside, and water connection. [c] Based on shedding and crushing. [d] Overall *P*-values ($P_{\text{Log Likelihood}}$) variable land use: for prevalence infected snails respectively P=0.24 and P=0.30 and for cercariae burden respectively P<0.01 and P<0.01. [e] Number of cercariae was missing for 51 positive snails.

Table 4. Number of snails shedding cercariae and median number of cercariae per snail with interquartile range per snail size class (mm).

Snail Size (mm)	Parapleurolophocercous cercariae per snail *N*	Median	Q1-Q3	Xiphidio cercariae per snail *N*	Median	Q1-Q3
<13[a]	18	20	9-107	18	34	7-154
13	13	56	19-176	17	48	5-247
14	22	44	25-94	38	64	9-216
15	29	83	8-262	37	150	36-374
16	30	51	20-401	24	104	33-455
17	25	71	42-260	17	116	3-273
18	36	83	23-209	26	181	62-378
>18[b]	73	76	34-258	46	207	26-571
Total	246	69	21-197	223	126	14-333

[a] average=11.6 mm; median=12 mm. [b] average=21.8 mm; median=21 mm.

Median burden of snails with parapleurolophocercous cercariae (n=246) was 69 (Table 4). Burden was associated with land use (P<0.01, Table 3); mean burden for all snails varied between 6 and 17 cercariae and was highest in the risk-category human access and livestock sty at one site (P<0.01). Of snails positive for xiphidio cercariae (n=223), median number of cercariae was 126 (Table 4). Burden was also associated with land use (P<0.01); it varied between 9 and 20 cercariae and was highest in the risk-categories human access (14 cercariae) and livestock sty (20 cercariae). For both cercariae types, burden increased significantly with increasing snail size (P<0.01) (Tables 3 and 4).

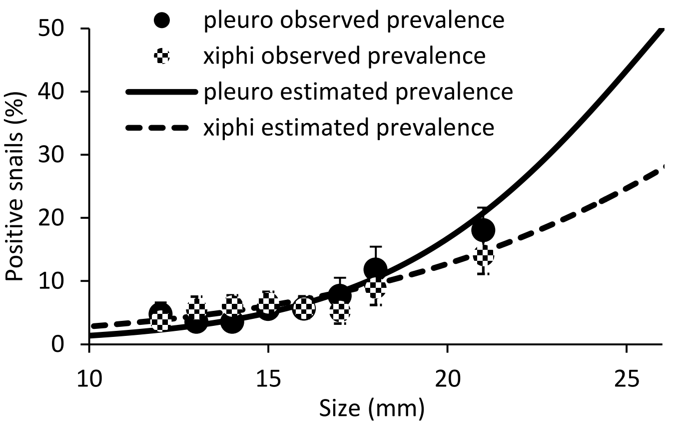

Figure 2. Estimated prevalence of parapleurolophocercous and xiphidio positive snails per mm size of *Melanoides tuberculata* snails based on univariable logistic regression. Equations for regression lines were 100/(1+ exp (6.9573 — 0.2676 x snail size (mm))), and 100/(1 + exp (5.1601 — 0.1618 x snail size (mm))) respectively. Observed prevalences with 95% exact confidence intervals are shown in size classes according to Table 4.

Discussion

In integrated small-scale aquaculture farming systems in Nam Dinh Province, Vietnam, prevalence of *M. tuberculata* snails with parapleurolophocercous and xiphidio cercariae was 6.6% for either cercariae type. Areas with different types of land use bordering that area surrounding a pond seem not to be associated with variation in trematode infection in snails for prevalence within small-scale aquaculture ponds. For parasite burden, there were significant differences, but one could argue whether a difference of about 11 cercariae is of biological importance, as cercariae burden can be up to 3470 for parapleurolophocercous cercariae per snail (Lo and Lee, 1996b). Because it has been reported that prevalence of snails with FZT was highest in small canals, compared to large canals, rice fields and fish ponds (Dung et al., 2010) a water connection was expected to be a risk factor, but this could not be demonstrated in our study.

The small variation within ponds imply that in field studies measuring prevalence of FZTs in snails, sampling from ponds can be done without considering areas within ponds. Observed prevalences might have been overestimated and distribution of cercariae burden per snail positively skewed, because snails with low cercariae count might be false-positive snails since they might have been contaminated by cercariae from other snails.

Parapleurolophocercous cercariae are produced by intestinal flukes (*Heterophyidae*) (Schell, 1985), of which especially *Haplorchis pumilio* is common in intermediate fish hosts, man and reservoir hosts in Nghia Lac, Nghia Phu and Nghia Hung communes, Nam Dinh province, Vietnam (Dung et al., 2007; Lan Anh et al., 2009a; Phan et al., 2010b). Xiphidio cercariae can be produced by e.g. *Plagiorchiidae* (Schell, 1985; Rogan et al., 2007). For example, the species *Plagiorchis muris* (*Plagiorchiidae*) that can be zoonotic (Hong et al., 1996), can have fish as second intermediate host (Chai and Lee, 2002), and has been observed in reservoir hosts in Nghia Lac and Nghia Phu communes, Nam Dinh province, Vietnam (Lan Anh et al., 2009a). Our overall prevalence of 13.8% was similar to that of Dung et al. (2010) who observed 13% of *M. tuberculata* infected with cercariae in July-September 2006 in the same area in Vietnam. Out of all positive snails, they found parapleurolophocercous and xiphidio cercariae in respectively 40 and 17% of positive snails, compared to 48% for both in our study. Furthermore, they reported echinostome and monostome cercariae in respectively 35% and 6% of positive snails, while we found these types in respectively only 2 and 1 out of 747 infected snails. Additionally, they have observed furcocercous, gymnocerphalous, and pleurolopho cercariae (together 1%), which we did not observe, and we have observed Transversotrema cercariae (4%), which Dung et al. (2010) did not observe.

Degree of infection of snails was associated with snail size, which was in accordance with a study from Lo and Lee (1996b). Other studies on trematodes reported the following two different hypotheses for this phenomenon. Jokela and Lively (1995) suggested a host-based explanation in which prevalence of infected snails depends on the time snails were exposed to infective stages of the trematodes. Genner et al. (2008) suggested a parasite-based explanation in which the parasite castrates its snail host resulting in gigantism of the snail. This phenomenon is common (Ben-Ami and Heller, 2008; Dillon, 2000; Sousa, 1983; Wright, 1971). Gigantism in snails may benefit trematodes by increasing cercariae output, thereby increasing transmission probability to the next host (Ebert, 1994; Genner et al., 2008).

Our observations show that infections of parapleurolophocercous and xiphidio cercariae in *M. tuberculata* snails within ponds only vary slightly with type of land use bordering a sampling area. This might be explained by: (1) free roaming reservoir hosts like dogs, cats and poultry (Anh et al., 2010a; Lan Anh et al., 2009a) could distribute trematodes eggs homogeneously around the pond so egg load might not vary between land use bordering a sampling area, and (2) the number of trematodes eggs might vary between land use bordering a sampling area and subsequently areas within ponds, but e.g. movement of snails might result in a homogenous distribution of infected snails in the pond (Snyder and Esch, 1993), as well as water movement by which egg load in the pond might be homogenized.

Reduced prevalence of FZTs in snails should lead to a reduced incidence of human infections. We did not observe snails in a duck enclosure, which was in accordance to a study reporting snail control by intensive farming of ducks (Levine, 1970). However, ducks can be final hosts for FZTs (Anh et al., 2010a) and might have reverse effects when introduced for intervention purpose.

To conclude, this study demonstrated little variation between sampling areas within a pond and areas with a different assumed risk surrounding a pond in risk of snails to be infected with cercariae. Snail control targeting specific areas within ponds is therefore not considered a potential intervention measure. Sampling from ponds for prevalence estimation of FZTs in snails can be done without considering areas within a pond.

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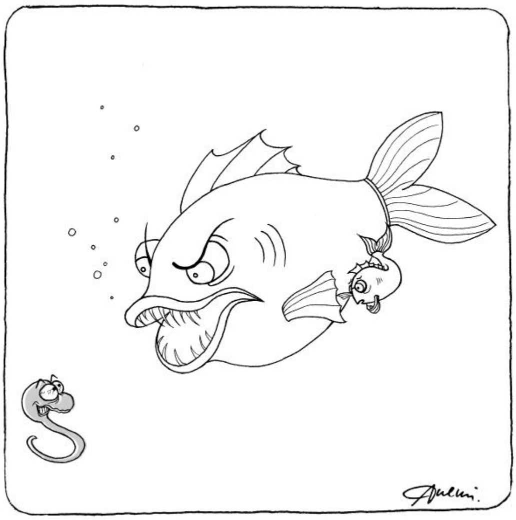
3

Effect of fish size on transmission of fish-borne trematodes (*Heterophyidae*) to common carp (*Cyprinus carpio*) and implications for intervention

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands



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Abstract

Fish-borne trematodes are reported to affect the health of more than 40 million people worldwide. Few experimental studies are available on fish size dependent gain (attack rates of cercariae) or loss (mortality of metacercariae) of fish-borne trematodes. Aim was to quantify the relation between fish size and attack rates of fish-borne trematodes in common carps (*Cyprinus carpio*). Effect of fish size and cercariae dose were tested in a 3x4 factorial design with 5 fish per combination of treatments (n=60). Individually kept small (1 g), medium (25 g) and large (45 g) carps were exposed to 0, 10, 50 or 250 parapleurolophocercous-cercariae (*Heterophyidae*) for 48 h. Fish were digested 21 days post exposure to count metacercariae. Percentages of fish containing metacercariae, and attack rates of cercariae to fish were higher (63%, 0.08 fish infected per cercariae) for small common carps than for medium (20%, 0.004 fish infected per cercariae) and large common carps (5%, 0.0007 fish infected per cercariae), but never zero. It was concluded that exposure of small fish is an important risk factor for transmission of fish-borne trematodes. The results suggest that control measurements aimed at reducing transmission to small fish may considerably reduce the absolute amount of fish-borne trematodes.

Keywords: fish-borne trematodes, transmission, fish size, intervention, common carp, metacercariae

Introduction

Fish-borne trematodes of the family *Heterophyidae* belong to the phylum *Platyhelminthes*, or flatworms. Life cycles of fish-borne trematodes involve three types of hosts. Primary aquatic snail hosts, secondary fish hosts and final hosts, like human, pigs and chicken (Sithithaworn et al., 2008). The health of more than 40 million people worldwide is affected by these trematodes, especially in South-East Asia and the Western Pacific (Abdussalam et al., 1995; WHO, 1995, 2002). About 70 species are known to be zoonotic (WHO, 2002), of which the most described species, *Opisthorchis viverrini*, *Clonorchis sinensis*, and *Opisthorchis felineus*, have been estimated to infect 17 million people (Sithithaworn et al., 2008). Raw or undercooked fish dishes are the major sources of human infection (Sithithaworn et al., 2008).

More than hundred fish species are susceptible to fish-borne trematodes (WHO, 1995). Between fish species, prevalence and parasite burden of C*. sinensis* are in general higher in small species compared to larger species (Komiya, 1966; Rim, 2005). Within species, young fish are assumed to be more susceptible to infection with fish-borne trematodes because of their relative thin skin and lack of previous exposure (reviewed by Lun et al., 2005). However, observational studies that take fish size into account were not consistent in their results. In larger fish, more trematodes were found by Kimura and Uga (2005). No relationship was found by Wang et al. (2002). Thien et al. (2007) observed several fish species and found both higher prevalence and higher burden in small fish compared to larger fish as well as no effects. They suggested that species-specific factors might be important for fish-borne zoonotic trematodes transmission. Thuy et al. (2010) observed higher prevalences with increasing age; fish weights were unknown. Phan et al. (2010c) found no infections in fish fry from hatcheries, and increasing prevalences from 14% infected one-week-old juveniles up to 58% in overwintered juveniles.

Observational studies, however, lack quantitative information about previous exposure of fish to cercariae. Prevalence and parasite burden of fish-borne trematodes in fish obtained at a specific point in time results from two processes: previous gain and loss of metacercariae. Fish gain metacercariae by a certain attack rate of cercariae to fish and lose metacercariae due to mortality of metacercariae in fish. Cercariae dose in water might be assumed to vary under normal pond conditions, since presence and amount of snail hosts vary among sites (Dung et al., 2010). Cercariae shedding by snails is known to be unpredictable and irregular (Schreiber and Schubert, 1949; Lo and Lee, 1996b). Furthermore, the effect of light, temperature, water flow and other environmental factors on cercariae shedding is not clear (Lo and Lee, 1996b; Phongsasakulchoti et al., 2005). This implies that for the relationship between metacercariae and fish size observed during observational studies attack rates of cercariae to fish cannot be estimated. Therefore, attack rates might be determined by experimental exposure only. In one experimental study, attack rates of cercariae of the eyefluke *Diplostomum spathaceum* were higher in rainbow trout (*Oncorhynchus mykiss*) of 5-7 cm than in rainbow trout of 12-15 cm when water volumes were proportional to fish surface areas (Hoglund, 1995). However, no other experimental studies are available on fish size dependent attack rates of cercariae or mortality of *Heterophyidae* metacercariae in fish.

Therefore, the aim of our experimental study was to quantify the effect of fish size on the percentage of fish with metacercariae and attack rates of fish-borne trematodes (*Heterophyidae*) in common carps after experimental exposure to different doses of cercariae.

Materials and methods

Experimental design, fish and parasites

Effect of fish size and cercariae dose was tested in a 3x4 factorial design with five fish per treatment (n=60). Common carps (*Cyprinus carpio*) were used as host fish because they are known as intermediate hosts for fish-borne trematodes and are used in traditional dishes (Phan et al., 2011; WHO, 1995). Small, medium and large common carps were purchased from one local fish farmer 6 days prior to start of the experiment. Age and history of fish was unknown. Until exposure, small, medium, and large fish were kept in two tanks containing 48 l well-water. Mean fish weight (SD) was 1.1 (0.4) g for small, 24.3 (4.8) g for medium, and 45.7 (8.8) g for large fish. Fish were fed commercial fish food at 5% body weight. No feed was provided from 24 h before to 12 h after exposure to cercariae.

Melanoides tuberculata snails were obtained one month prior to the experiment from households in Ngia Lac, Nam Dinh Province, Vietnam, where a high prevalence of *Heterophyidae* was observed (Anh et al., 2010a; Dung et al., 2007, 2010; Phan et al., 2010c). Snails were individually kept in glass cups filled with about 5 ml well-water for 2 h to shed cercariae. Most *Heterophyidae* cercariae are parapleurolophocercous-cercariae (Schell, 1985), therefore only snails shedding parapleurolophocercous-cercariae were used. Cercariae were identified and counted under a stereomicroscope as they were drawn into a pipet. Pipets were emptied in petri-dishes and number of cercariae was recounted. Petri-dishes containing 10, 50 or 250 cercariae were emptied in tanks with a bottom surface of 11 x 11 cm containing 500 ml well-water.

Fish were kept and exposed individually to 0, 10, 50 or 250 cercariae for 48 h, all at the same date. During exposure, an air-stone was provided to each tank. After exposure, fish were dipped in well-water for 1 minute to wash remaining cercariae off the fish. During 21 days after exposure, fish were individually kept in net cages in tanks containing 3000 l of cercariae free water. Water temperature was recorded daily, and was on average 21ºC, varying between 18 and 22. Every tank contained 12 net cages containing one fish each. Dead fish were collected two times daily, and stored at 5ºC until digestion.

Parasite recovery

Metacercariae were recovered from fish within 36 h post mortem or at the end of the experiment 21 days after exposure to cercariae. Before digestion, weight and total length of the fish were recorded. Digestion protocol was an adjusted version of the digestion method described in annex 6 of WHO (1995). Fish were individually grinded in artificial stomach acid solution consisting of 0.06 M HCl and 1% pepsin in distilled water, with a ratio of 100 g of fish per liter artificial stomach acid. The mixture was incubated for 2-3 h at 37ºC, after which the digested material was filtered through a 1 x 1-mm mesh brass sieve and washed several times with 0.85% saline solution until the supernatant became clear. The sediment was searched twice by stereomicroscope. Recovered metacercariae were collected, counted and identified by microscope at 500x magnification, based on morphological characteristics described by

Scholz et al. (1990; 1991) and according to the FIBOZOPA laboratory manual (2006, unpublished).

Statistical analysis
Survival analysis was used to study the effect of fish size and cercariae dose on survival time of fish. Survivor functions were estimated using the Kaplan-Meier method, and compared with the log rank test (PROC LIFETEST, SAS Inst. Inc., 2004).

Infection (yes/no) was analyzed by (exact) logistic regression (PROC LOGISTIC, SAS Inst. Inc., 2004) in which effect of fish size (small, medium, large), cercariae dose (0, 10, 50, 250), and their interaction were tested. A fish was considered infected when at least one metacercariae was recovered. Results were expressed as prevalences and Odds Ratios. An Odds Ratio (OR) is a measure of the association between exposure and infection; an OR>1 indicates that a variable increases the risk of infection; an OR<1 that it decreases the risk (Noordhuizen *et al*., 2001).

The number of recovered metacercariae was analyzed with generalized linear models with a Poisson distribution and log link function (PROC GLIMMIX, SAS Inst. Inc., 2004). Effect of fish size, infection dose, and their interaction were included in the model as explanatory variables.

For undeveloped metacercariae, we calculated the attack rate in two ways. First, using the number of cases (fish with metacercariae), second using metacercariae burden.

In the first method, data were statistically analyzed using generalized linear models to estimate the attack rate λ. A binomial distribution with a complementary-log-log link function was used, with offset:
log(cercariae exposure dose+1) (PROC GENMOD, SAS Inst. Inc., 2004). Expected number of fish with metacercariae (E(C)) is then equal to:

$$E(C) = (1\text{-}\exp[-\lambda \bullet (\text{cercariae exposure dose}+1)]) \bullet S,$$

or

$$E(C/S) = (1\text{-}\exp[-\lambda \bullet (\text{cercariae exposure dose}+1)]),$$

in which S is the number of susceptible fish at start of the experiment.

Taking the log of this model results in:

$$\log E(C/S) = \log(\lambda) + \log(\text{cercariae exposure dose}+1).$$

Fish size (small, medium, large) was included in the model as fixed effect. The estimated parameter is $\log(\lambda)$; exponentiation gives λ and represents the number of infected fish caused by one cercariae as result of exposure. Controls (infection dose=0) were excluded because transmission is not possible.

Second, attack rate based on metacercariae burden was defined as the number of new metacercariae recovered per cercariae of the exposure dose. Data were analyzed using nonparametric models because metacercariae burden was not normally distributed. Wilcoxon scores were used to rank the observations for fish size (small, medium, large) (PROC NPAR1WAY, SAS Inst. Inc., 2004).

Results

Survival

In total, 97% of fish survived 48 h of exposure to cercariae. At 21 days post exposure, survival was 38% with resp. 0, 50, and 63% for small, medium, and large fish (Table 1). Survival curves did not differ between infection doses ($P=0.98$). Mean survival time ranged from 13 to 16 days. Survival curves differed between fish sizes ($P<0.01$), mean survival time was 9 days for small and 18 days for medium and large fish.

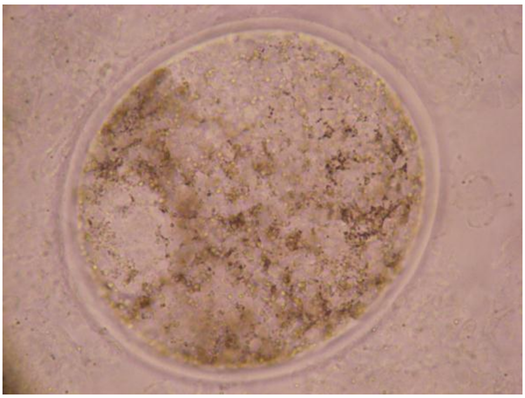

Figure 1. Undeveloped metacercariae 21 days post exposure.

Table 1. Survival of fish after 48 h of exposure to cercariae and at 21 days post exposure to parapleurolophocercous-cercariae, mean survival times of fish, and P-values of equality among strata.

	Effect	Sample size at start of the experiment	Number of fish alive after 48 h exposure (% survival)	Number of fish alive at 21 days p.i. (% survival p.i.)	Mean survival time in days (± se)	P-values equality among strata (log-rank)
Overall		60	58 (97%)	22 (38%)	15.28 (±0.73)	
Fish size	Small	20	19 (95%)	0 (0%)	9.45 (±0.99)	<0.0001
	Medium	20	20 (100%)	10 (50%)	18.05 (±0.65)	
	Large	20	19 (95%)	12 (63%)	17.75 (±0.90)	
Cercariae dose (no. cercariae)	0	15	15 (100%)	5 (33%)	15.73 (±1.34)	0.9822
	10	15	14 (93%)	5 (36%)	14.67 (±1.59)	
	50	15	14 (93%)	7 (50%)	12.73 (±1.28)	
	250	15	15 (100%)	5 (33%)	15.80 (±1.33)	

Identification, prevalence, and cumulative incidence

Recovered metacercariae were divided in 3 morphologically distinct groups, i.e. *Haplorchis*, *Centrocestus*, and an undeveloped undefined group. Identification of the undeveloped metacercariae by morphologic characteristics was unsuccessful, because of its formless, motionless look in which no organs could be distinguished (Fig. 1).

Percentage of fish with *Haplorchis* varied from 7 to 27% between exposure doses, and was not significantly different (P=0.50). Percentage of fish with *Centrocestus* varied from 27 to 43%, and was also not different between exposure doses (P=0.64). Both *Haplorchis* and *Centrocestus* were found in the control group, indicating the presence of these metacercariae at the start of the experiment. On the contrary, undeveloped metacercariae were absent in control fish. Compared to control fish with a percentage of 0, the percentage of fish with undeveloped metacercariae increased with exposure (P<0.01), and was 21% in fish exposed to 10 cercariae (OR=4.7; P=0.20), 43% in fish exposed to 50 cercariae (OR=13.0; P=0.01), and 53% in fish exposed to 250 cercariae (OR=19.7; P<0.01) (Table 2).

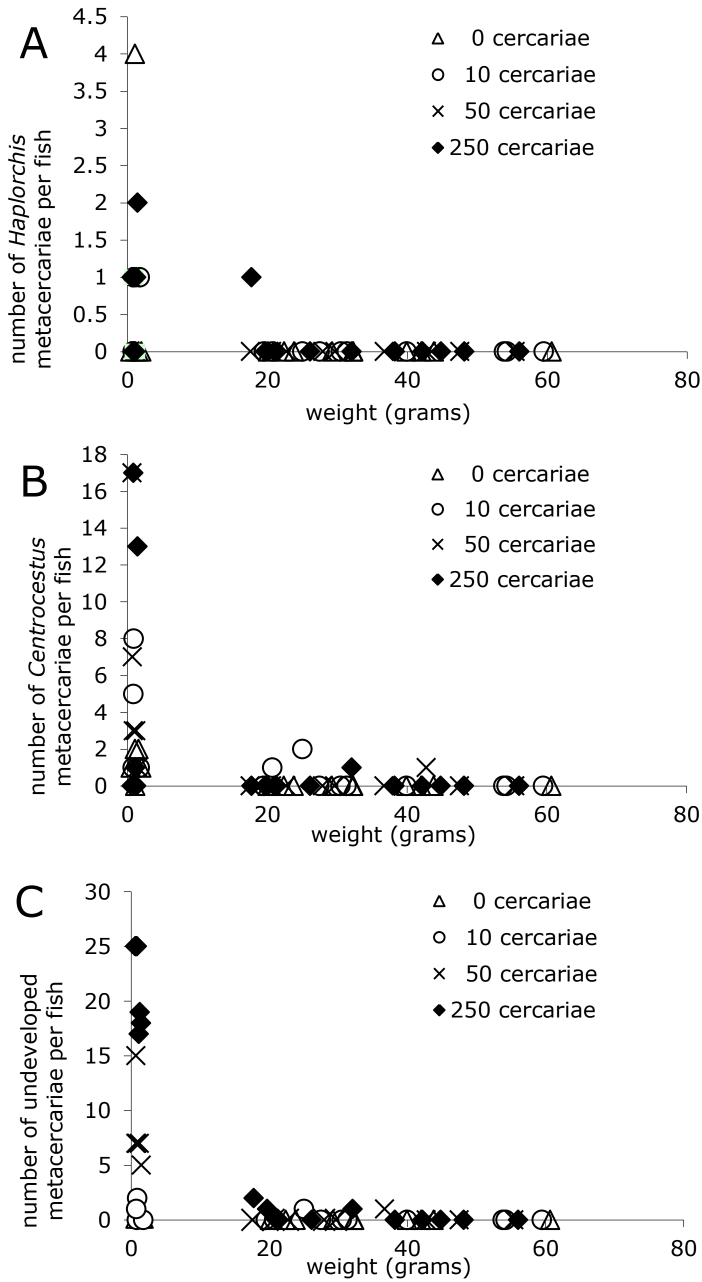

Figure 2. Relation between fish weight (grams) and number of recovered metacercariae per fish after exposure to 0, 10, 50 or 250 parapleurolophocercous-cercariae. A: *Haplorchis* metacercariae, B: *Centrocestus* metacercariae, and C: undeveloped metacercariae.

Percentages of fish with metacercariae were significantly lower in medium and large fish compared to small fish for the three different species of metacercariae (P<0.01). Compared to the large fish with a percentage of 0%, the percentage of fish with *Haplorchis* was not different with 5% for medium fish size (OR=1.0; P=1.00), but was higher for small fish with 42% of fish with metacercariae (OR=17.0; P<0.01). For *Centrocestus* these percentages differed between fish size, with resp. 5% for large fish, 15% for medium (OR=3.2; P=0.64), and 84% for small fish (OR=77.0; P<0.01). For undeveloped metacercariae, the percentage was 5% in large fish compared to 20% in medium fish (OR=4.5; P=0.20), and compared to 63% in small fish (OR=31; P<0.01) (Table 3).

Table 2. Prevalence (Prev), Odds Ratio, 95% confidence interval, and P-values of fish positive for *Haplorchis*, *Centrocestus*, and undeveloped metacercariae after experimental exposure to different parapleurolophocercous-cercariae doses.

Species Metacercariae	Cercariae dose (no.)	Number of fish	Prev (%)	Odds Ratio	95% CI	P_{Wald}	$P_{Likelihood\ Ratio}$
Haplorchis	0	15	6.7	ref.			0.5028
	10	14	14.3	2.33	0.19-29.02	0.5102	
	50	14	14.3	2.33	0.19-29.02	0.5102	
	250	15	26.7	5.09	0.50-52.25	0.1709	
Centrocestus	0	15	26.7	ref.			0.6397
	10	14	42.9	2.06	0.43-9.80	0.3628	
	50	14	42.9	2.06	0.43-9.80	0.3628	
	250	15	26.7	1.00	0.20-5.05	1.0000	
Undeveloped	0	15	0.0	ref.			0.0056[b]
	10	14	21.4	4.67[a]	0.47-∞	0.1992[b]	
	50	14	42.9	13.03[a]	1.63-∞	0.0126[b]	
	250	15	53.3	19.73[a]	2.58-∞	0.0022[b]	

[a]: median unbiased estimate [b]: exact test

Parasite burden and attack rates

Minimum, medians (interquartile ranges), and maximum number of recovered metacercariae per fish for small (n=19); medium (n=20); and large fish (n=19) were for *Haplorchis* 0, 0 (0-1) 4; 0, 0 (0-0) 1; and 0, 0 (0-0) 0 (Fig. 2A). For the number recovered *Centrocestus* metacercariae per fish these were resp. 0, 1 (1-7) 17; 0, 0 (0-0) 2; and 0, 0 (0-0) 1

(Fig. 2B), and for undeveloped metacercariae resp. 0, 5 (0-17) 25; 0, 0 (0-0) 2; and 0, 0 (0-0) 1 (Fig. 2C).

Effect of infection dose and fish size on metacercariae burden was similar for recovered *Haplorchis* and *Centrocestus* metacercariae. Exposure dose had no effect on parasite burden (resp. P=0.79 and P=0.40), whereas fish size had (both P<0.01). No interactions between exposure dose and fish size were observed (resp. P=0.99 and P=0.50). Undeveloped metacercariae burden in fish, however, was associated independently with both exposure dose (P<0.01) and fish size (P<0.01); the interaction was not significant (P=0.88).

Both estimations of attack rates of parapleurolophocercous-cercariae showed similar results. Estimated attack rate based on number of cases (95% confidence interval), i.e. number of infected fish per cercariae exposed to was different (P<0.01) for small, medium and large fish, resp. 0.08 (0.03-0.24), 0.004 (0.00003-0.05), and 0.0007 (0.00002-0.02). Based on metacercariae burden these outcomes were 0.11 (0.08), 0.008 (0.03), and 0.001 (0.005) for small, medium and large fish respectively (P<0.01).

Table 3. Prevalence (Prev), Odds Ratio, 95% confidence interval, and P-values of fish positive for *Haplorchis*, *Centrocestus*, and undeveloped metacercariae in small (1 g), medium (25 g) and large fish (45 g) after exposure to 0, 10, 50 or 250 parapleurolophocercous-cercariae per fish.

Species metacercariae	Fish size	Number of fish	Prev (%)	Odds Ratio	95% CI	P_{Wald}	$P_{Likelihood\ Ratio}$
Haplorchis	Small	19	42.1	16.87[a]	2.31-∞	0.0031[b]	
	Medium	20	5.0	0.95[a]	0.02-∞	1.000[b]	
	Large	19	0.0	ref.			0.0005
Centrocestus	Small	19	84.2	77.01	7.72-∞	<0.0001	
	Medium	20	15.0	3.09	0.22-175.85	0.6445	
	Large	19	5.3	ref.			<0.0001
Undeveloped	Small	19	63.2	30.85	3.36-283.70	0.0025	
	Medium	20	20.0	4.50	0.46- 44.53	0.1985	
	Large	19	5.3	ref.			0.0002

[a]: median unbiased estimate [b]: exact test

Discussion

Aim was to quantify the effect of fish size on the percentage of fish with metacercariae and attack rates of fish-borne trematodes to common carps. We observed a higher percentage of infected small fish and a higher attack rate to small fish compared to medium and large fish.

Cercariae species could not be distinguished based on morphology. We assumed that parapleurolophocercous-cercariae used for exposure were of the genus *Haplorchis*, because the species *H. pumilio, H. taichui*, and *H. yokogawai*, all parapleurolophocercous-cercariae, were the most prevalent species in the area where snails were collected (Dung et al., 2007; Lan Anh et al., 2009b; Phan et al., 2010c). *H. pumilio* and *C. sinensis* metacercariae in fish were reported identifiable 14 days post cercariae invasion (Komiya, 1966; Umadevi and Madhavi, 2006). In our experimental design, all fish were digested maximum 21 days post exposure; firstly, because metacercariae were expected to be fully developed and identifiable, and, secondly, to prevent possible bias of metacercariae loss in fish. At 21 days post exposure, we observed *Haplorchis, Centrocestus and a* group of undeveloped metacercariae of which the body was a formless, motionless mass without clear organs. These formless metacercariae corresponded to pictures and descriptions of early metacercariae stages of *C. sinensis* (Komiya, 1966), seemed smaller than the observed *Haplorchis* and *Centrocestus* metacercariae, and moreover was absent in control fish. However, *Haplorchis* and *Centrocestus* metacercariae were present in control fish in numbers not different from experimentally exposed fish. Analysis showed that fish experimentally exposed to cercariae had no increased risk of being infected with *Haplorchis* or *Centrocestus* metacercariae, but had an increased risk of being infected with undeveloped metacercariae. Also, percentage of fish with undeveloped metacercariae increased at increasing cercariae dose. We therefore assumed that *Haplorchis* and *Centrocestus* were already present before experimental exposure and undeveloped metacercariae were gained by experimental exposure to cercariae. Given the fact that in the control group of fish that were not exposed to cercariae, in not a single fish undeveloped metacercariae were found, we deemed it reasonable to make this assumption. Theoretically, the effect of experimental exposure dose could have been influenced by the already present *Haplorchis* and *Centrocestus*, because of interactions between the different species. This was, however, considered of minor importance as infection level with *Haplorchis* and *Centrocestus* was similar for all fish in the study population, which should make comparison of fish of different sizes valid.

Infection dynamics of metacercariae in fish consists in general of two main processes; gain by cercariae attack rate, and loss by metacercariae mortality. Regarding metacercariae gain, we observed higher attack rates of parapleurolophocercous-cercariae and higher percentages of fish with undeveloped metacercariae in small fish compared to medium and large fish. Both methods of estimating attack rates, i.e. based on number of infected fish and metacercariae burden, were comparable in magnitude, and considered valid. Previous exposure dose of fish to cercariae of *Haplorchis* and *Centrocestus* was unknown, therefore attack rates for these parasites could not be estimated. Possible reasons for higher attack rates in small fish compared to medium and large fish might be attributed to differences in quantity or composition of biochemic compounds excreted by fish that might change when fish grow (Haas, 1992). Age-related differences in immunological defence of common carp are shown during the first weeks post fertilization, when the immune system is developing (Huttenhuis et al., 2006). Common carp might grow to 1 g within 4 weeks when growth is efficient (Huisman, 1974). Therefore, the adaptive immune system of our small fish, having an estimated age of 50-60 days, could be less developed compared to medium and large fish, which had an approximate age of 4 to 6 months. As exact age of our fish was not known, conclusions about age-related effects cannot be drawn.

This study shows that gain of metacercariae in fish depends on fish size. To estimate the effect of size on occurrence of infection and parasite burden of a fish population in time, we need to know both gain and loss rates of metacercariae in fish at a specific point in time. Unfortunately, not much is known about loss of metacercariae in fish. Fried et al. (2004) reported that metacercariae in fish are viable for years. Another study presented evidence of metacercariae loss 50 days after infection (Mitchell et al., 2002). Encysted *Clonorchis sinensis* metacercariae in freshwater minnow (*Zacco platypus*) degenerated at 10 weeks, which was a reason for Komiya (1966) to exclude freshwater minnow as a host for *C. sinensis*, thereby indicating that life-span of metacercariae in fish is in general longer. Although we found a longer survival time and lower metacercariae burden of medium and large fish compared to small fish, we cannot conclude anything about metacercariae loss rates as these were counted at 21 days at the latest.

Reduction of exposure of humans to fish with lower metacercariae burden should lead to a reduced incidence of human infection with fish-borne trematodes. Although our experiment was done with intestinal flukes, this most probably helps us in understanding the life-cycle of liver flukes, which are more harmful to humans than intestinal flukes (Lima dos Santos and Howgate, 2011). Fish size matters, but what are the implications with regard to sustainable intervention in aquaculture? Intervention can be implemented on many different levels and hosts. Humans act as final host and goal of prevention can be reducing infection in humans by preventing them from eating raw or undercooked fish. However, reservoir hosts, like pigs, dogs, cats, fish-eating birds and ducks (Anh et al., 2010a; Lan Anh et al., 2009b; Lun et al., 2005), might transmit trematodes and continue the life cycle, in which case there is still a risk for humans to get infected. Control measurements aiming at reducing transmission to small fish may reduce a large portion of the absolute amount of fish-borne trematodes in the environment. Changing management practices, like hatching fry in cement or composite tanks with filtered water, might serve as an effective control measurement as studies of Thien et al. (2009) and Phan et al. (2010c) found lack of infection in their studies in which fry were raised in tanks with filtered water free of zooplankton, snails and other microbiota. Our study does not provide guidelines for an exact fish size that can be used as a threshold in intervention practices, because attack rate to fish of 1 g is much higher than to fish of more than 20 g. The relation between attack rates and fish size for fish smaller than 20 g might be a linear decrease, but might also be an abrupt decrease at a certain size, which might have implications for intervention, and should therefore be studied in more detail.

To conclude, this study demonstrates the importance of fish size as risk factor for transmission of fish-borne trematodes in general.

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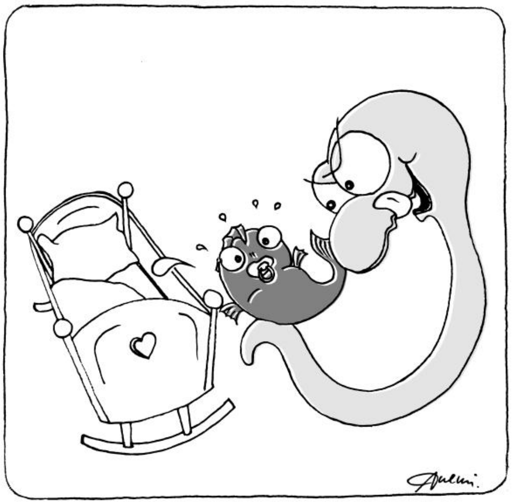
4

Higher attack rate of fish-borne trematodes (*Heterophyidae*) in common carp fingerlings (*Cyprinus carpio*) at lower fish weight

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands



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Abstract

Fish-borne zoonotic trematodes (FZTs) can cause pathology in humans. Fish weight was reported as important risk factor for transmission from snail to fish. However, in fingerlings, the relation between fish weight and infection is unknown. Aim was quantifying the effect of fish weight on infection probability, attack rate and metacercariae burden of FZTs in common carps (*Cyprinus carpio*) between 1 and 20 g. Fish were either used as controls (n=66) or exposed to 250 parapleurolophocercous cercariae (n=254). Fish weight was analysed as continuous explanatory variable or classified in four categories with average weights of 0.7 (n=116), 4.0 (n=58), 8.2 (n=57), and 14.2 g (n=23). The inverse relation between percentage of fish with metacercariae and fish weight is reflected in lower percentages of infected fish at higher weights [%infected=100/(1+e[-2.02+0.15 fish weight (g)]); $p<0.01$], i.e.. 89 %, 85 %, 63 % and 61%, respectively, in the four groups. Control fish did not get infected. Attack rates were 0.0087, 0.0073, 0.0040 and 0.0033 fish infected per cercariae, respectively; the first two attack rates being significantly higher than the latter two. Mean number of metacercariae per weight group was 5, 5, 2 and 1, respectively, ($p<0.05$), with an inverse relation using weight as continuous explanatory variable [$p<0.01$; number metacercariae=exp[1.76-0.13 fish weight(g)]. Concluding, an inverse relation exists between fish weight and probability of infection, attack rate and parasite burden in common carp fingerlings. Reducing transmission to fingerlings might be an effective intervention method to improve food safety, reduce the absolute amount of FZTs in the environment, and eventually reduce incidence in humans.

Keywords: fish-borne trematode, transmission, fish weight, common carp, metacercariae

Introduction

Fish-borne zoonotic trematodes (FZTs) are among the most neglected tropical disease agents (WHO, 2011). Main intervention strategy against liver and lung flukes, which pose the most significant health burden, is prophylaxis to the human population at risk (WHO, 2011). In general, intestinal flukes cause less severe pathology in humans (Toledo et al., 2006), but have become increasingly important because of food safety requirements (Chai et al., 2005). Therefore, and also considering the life-cycle of FZTs that involves primary intermediate snail hosts, secondary intermediate fish hosts, and final hosts like humans, pigs, dogs, cats, or fish eating birds (Anh et al., 2010a; Dung et al., 2010; Lan Anh et al., 2009a; Phan et al., 2010c; Phan et al., 2011), intervention targeting the fish host in aquaculture settings might be an effective strategy.

One of the factors not always consistent in the increasing number of observational studies (e.g. Chai et al., 2005; Keiser and Utzinger, 2005; Lima dos Santos and Howgate, 2011) is the relation between fish weight and infection probability and/or attack rates in FZTs. An experimental study showed that small common carp (1 g) after exposure to FZTs were more often infected and cercariae had higher attack rates to fish of 1 and 20 g than to fish of 45 g. It was concluded that fish weight is an important risk factor for attack rates of FZTs in fish (Boerlage et al., 2011). A lower attack rate and thus lower transmission to fish may reduce the incidence of human infection. The relation between attack rates and fish weight in 1-20 g fish might be gradual or abrupt, in which case an absolute threshold weight for intervention can be estimated. Aim of this experiment was to quantify the relation between fish weight and infection probability, parasite burden, and attack rate of FZTs in common carps between 1 and 20 g.

Materials and methods

In October 2010, common carps (*Cyprinus carpio*), purchased from one local fish farmer 2 months prior to exposure, were kept in two tanks containing 1250 l well water. Age and history of fish was unknown. Ten days prior to exposure, fish of about 0.5, 1, 5, 10, 15, 20 g were selected and moved into six tanks, each containing 80 l well water and two air stones. Fish were fed commercial fish food at 5% body weight. No feed was provided from 24 h before to 12 h after exposure to cercariae. *Melanoides tuberculata* snails were obtained 5 months prior to the experiment from households in Ngia Lac, Nam Dinh Province, Vietnam.

Protocols for snail shedding, fish exposure, fish digestion, and metacercariae recovery were according to Boerlage et al. (2011). Shortly, snails were individually kept for 8 h to shed parapleurolophocercous cercariae, which are the larval stage of intestinal flukes (Schell, 1985). Twelve hours after onset of cercariae shedding, individual fish were exposed to 250 or 0 (control) cercariae for 24 h in exposure tubes with a diameter of 85 mm containing 200 ml well water. Exposure was performed during 11 different days in a total period of 45 days. Each exposure day, six fish were kept as control (n=66). The number of exposed fish depended on the amount of cercariae available after snail shedding and was 21, 33, 37, 11, 6, 20, 14, 22, 35, 35 and 20, respectively, per exposure day (n=254). During 1 week after exposure, fish were individually kept in glass beakers containing 500 ml well water and, after that, weighted, digested and metacercariae counted. A fish was considered infected when at least one metacercaria was recovered.

For statistical analysis, fish weight was either used as a continuous explanatory variable or class variable classified in four weight groups: fish up to 1 g (weight I), about 5 g (weight II), about 10 g (weight III) and between 10 and 22 g (weight IV). Infection (yes/no) was analysed by generalised linear models with a binomial distribution, and a logit link function (PROC GENMOD, SAS Institute Inc., 2008) in which cercariae dose (0 or 250) and fish weight were included as explanatory variables. Attack rate, i.e. the number of fish infected per exposed cercariae, were estimated based on infection (yes/no) using generalised linear models with a binomial distribution, a complementary log-log link function and an offset of log(cercariae exposure dose+1); (PROC GENMOD, SAS Institute Inc., 2008) similar to Boerlage et al. (2011). Weight group was included in the model as explanatory variable. The relation between weight and number of metacercariae recovered per fish was analysed with generalised linear models using a Poisson distribution and log link function (PROC GENMOD, SAS Institute Inc., 2008).

Observations per exposure day cannot be regarded independent. Therefore exposure day was included as random effect using an exchangeable correlation structure in the models described above, except when probability of infection was zero. Then an exact test was used instead.

Results

Recovered metacercariae were identified as *Haplorchis*, *Centrocestus*, and an undeveloped group. *Haplorchis* metacercariae had an O-shaped excretory bladder and minute spines around the ventrogenital complex (Scholz et al., 1990; Fig. 1A). *Centrocestus* metacercariae had an X-shaped excretory bladder and circumoral spines around the oral sucker (Scholz et al., 1990; Fig. 1B). Undeveloped metacercariae could not be identified because of their formless, motionless look in which no organs could be distinguished, identical to observations in Boerlage et al. (2011; Fig. 1C).

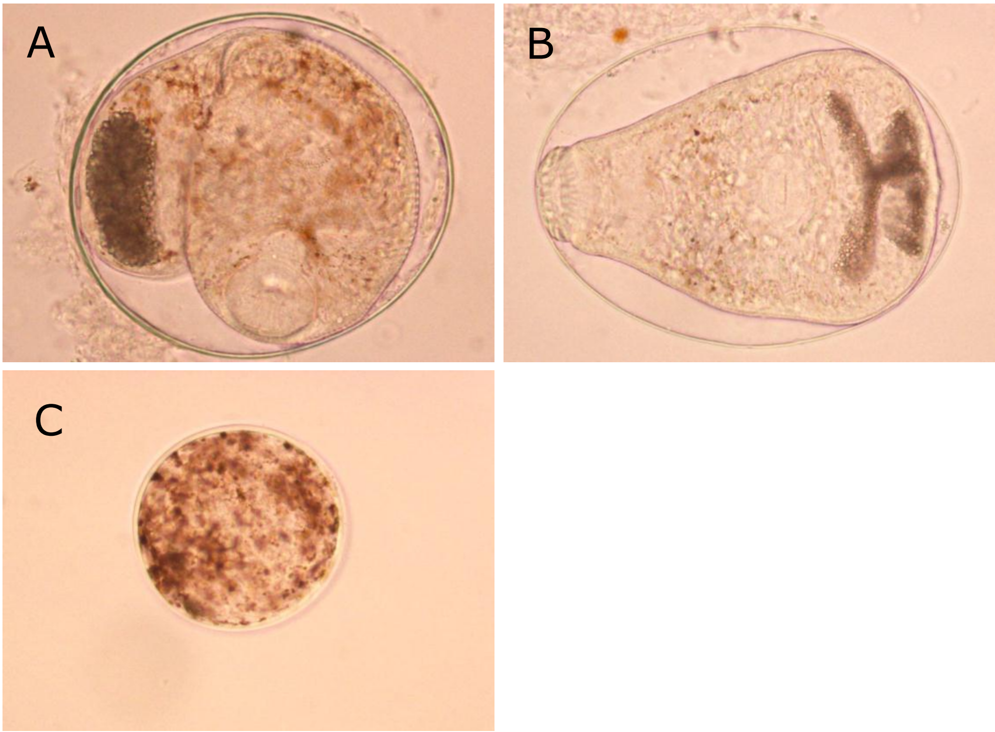


Figure 1A. Haplorchis metacercariae, 1B. Centrocestus metacercariae. 1C. Undeveloped metacercariae.

Both *Haplorchis* and *Centrocestus* were present in control fish, indicating the presence of these metacercariae at the start of the experiment. Percentage of infected fish with these types (2 % and 29 %, respectively) in control fish was different from exposed fish (resp. 14 % and 56 %, respectively; $p<0.01$). Undeveloped metacercariae were absent in control fish and were recovered from 80% of exposed fish ($p<0.01$; exact logistic regression).

Mean fish weight (SD) for controls ($n=66$) and weight groups I ($n=116$), II ($n=58$), III ($n=57$) and IV ($n=23$) fish was 2.8 (2.6) g, 0.7 (0.2) g, 4.0 (0.4) g, 8.2 (1.0) g and 14.2 (3.4) g, respectively, (Table 1). Percentage of *Haplorchis* in exposed fish was lower in group I (3%) than in group IV (87%) ($p<0.05$). Also percentage of fish with *Centrocestus* in exposed fish was lower at low weight in group I (47%) compared to group IV (83%) ($p<0.05$). Conversely, percentage of fish with undeveloped metacercariae in exposed fish differed from 89% in group I to 61% in group IV ($p<0.05$); group I and II being significantly more often infected than group III and IV (Table 1). Exposure day explained 1 %, 2 %, and 13% of unexplained variance in infection probability for *Haplorchis, Centrocestus*, and undeveloped metacercariae, respectively.

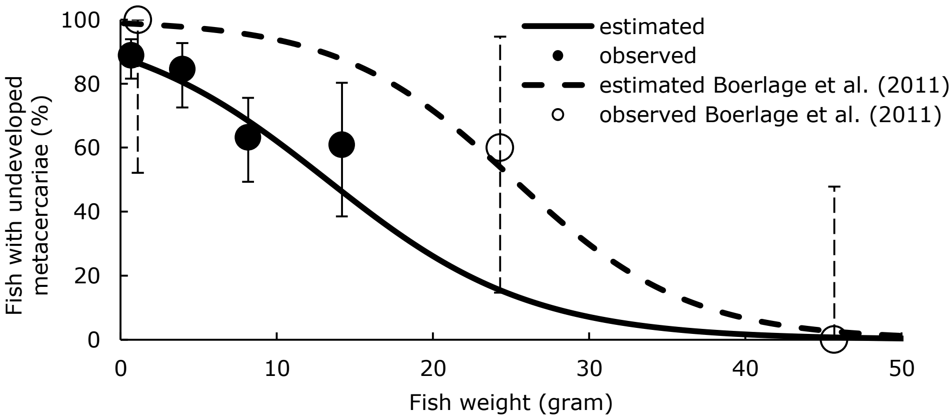


Figure 2. Observed and estimated percentages of infected fish of this experiment and from Boerlage et al. (2011; cercariae dose 250 only) of FZTs in common carp related to fish weight (g) based on logistic regression with correction for exposure day. Estimated percentage of infected fish for this experiment with weight as continuous explanatory variable ($n=254$): 100/(1+exp[-2.0214+0.1531 fish weight (g)]) and for Boerlage et al. (2011): 100/(1+exp[-4.4854+0.1781 fish weight (g)]) ($n=15$). Observed prevalences with 95% exact confidence intervals are shown per weight group.

A significant inverse relation was shown between the expected percentage of fish with metacercariae and fish weight (p<0.01; Fig. 2). Comparison with Boerlage et al. (2011) for fish with an exposure dose of 250 cercariae shows that, in both experiments, the infection probability in fish of 50 g is almost zero; however, in the current experiment, the regression coefficient for weight was smaller (Fig. 2). Estimated attack rates were 0.0087, 0.0073, 0.0040 and 0.0033 for the weight groups I-IV, respectively, (p<0.05). Exposure day explained 13% of unexplained variance (Table 1).

Mean number of metacercariae per fish was different for the weight groups: 0, 0, 3 and 19, respectively, (p<0.05) for *Haplorchis*, 1, 1, 7, 25, respectively, (p<0.05) for *Centrocestus* and 5, 5, 2 and 1, respectively, (p<0.05) for undeveloped metacercariae (Table 1). Fish in weight group IV showed higher numbers of *Haplorchis* and *Centrocestus* metacercariae than in other weight groups (p<0.05). The number of undeveloped metacercariae were lower in weight groups III and IV compared to weight groups I and II (p<0.05). Exposure day explained 2 %, 3 % and 37 % of unexplained variance in metacercariae burden for *Haplorchis, Centrocestus*, and undeveloped metacercariae, respectively. The expected number of undeveloped metacercariae per fish after exposure to 250 cercariae showed an inverse relation with fish weight (p<0.01) similar to Boerlage et al. (2011), but the expected values were lower in current experiment (Fig. 3).

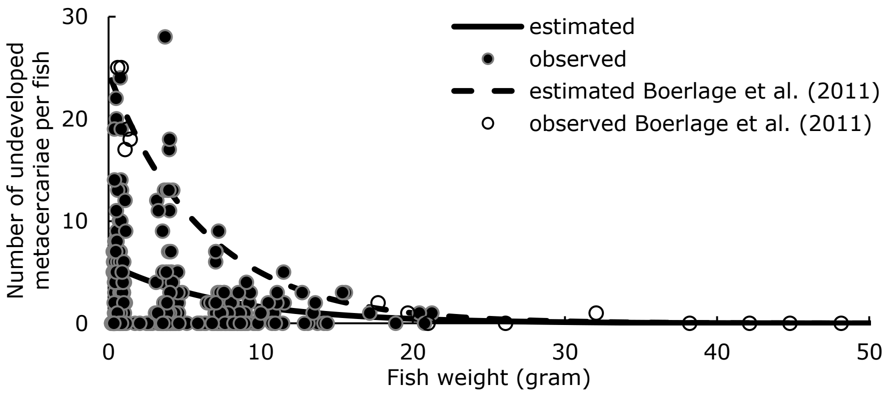


Figure 3. Observed and estimated number of undeveloped metacercariae per fish of this experiment and from Boerlage et al. (2011; cercariae dose 250 only) related to fish weight (g) based on Poisson regression. Estimated number of metacercariae for this experiment with weight as continuous explanatory variable: exp[1.7647-0.1283 fish weight (g)] (n=254), and for Boerlage et al. (2011): exp[3.2012-0.1596 fish weight (g)] (n=15).

Table 1. Number of fish, average fish weight (g±SD), percentage of infected fish and lsmeans (SEM) of parasite burden for *Haplorchis, Centrocestus* and undeveloped metacercariae, and attack rates (=fish infected per cercariae) (95% CI) of undeveloped metacercariae per weight group of fish exposed to 250 metacercariae.

Weight group	No. fish	Average weight (g±SD)	Percentage of fish infected with *Haplorchis*	Percentage of fish infected with *Centrocestus*	Percentage of fish infected with Undeveloped	*Haplorchis* lsmeans (SEM) burden/fish	*Centrocestus* lsmeans (SEM) burden/fish	Undeveloped lsmeans (SEM) burden/fish	Undeveloped attack rate (95% CI)
I	116	0.7 (±0.2)	2.6^{a}	47.4^{a}	88.8^{a}	$0.0 (0.02)^{a}$	$0.8 (0.11)^{a}$	$5.1 (0.95)^{a}$	$0.0087 (0.0066\text{-}0.0114)^{a}$
II	58	4.0 (±0.4)	5.2^{ab}	50.0^{ab}	84.5^{a}	$0.1 (0.06)^{a}$	$1.0 (0.19)^{a}$	$4.5 (1.45)^{a}$	$0.0073 (0.0051\text{-}0.0104)^{a}$
III	57	8.2 (±1.0)	17.5^{b}	66.7^{bc}	63.2^{b}	$2.8 (0.42)^{ab}$	$7.2 (1.33)^{ab}$	$1.6 (0.33)^{b}$	$0.0040 (0.0029\text{-}0.0055)^{b}$
IV	23	14.2 (±3.4)	87.0^{c}	82.6^{c}	60.9^{b}	$19.2 (1.39)^{bc}$	$24.6 (2.77)^{bc}$	$1.2 (0.41)^{b}$	$0.0033 (0.0017\text{-}0.0061)^{b}$

^{abc}: Different superscripts within columns indicate significant differences ($p<0.05$).

Discussion

Fish were acclimatised 2 months in well water before experimental exposure. *Haplorchis* and *Centrocestus* metacercariae that were already present in fish before exposure had, in this period, the opportunity to become mature and identifiable (Komiya, 1966; Umadevi and Madhavi, 2006). Time from exposure to digestion of 7 days was considered short enough to distinguish undeveloped metacercariae from developed metacercariae that were present in fish before experimental exposure (Boerlage et al., 2011) and long enough for undeveloped metacercariae to develop a cyst (Komiya, 1966) to protect the metacercariae during digestion. Undeveloped metacercariae were absent in control fish and corresponded to descriptions of undeveloped metacercariae in Boerlage et al. (2011).

We assumed that all undeveloped metacercariae resulted from experimental exposure and therefore we estimated attack rates based on undeveloped metacercariae only. Because percentages of infected fish and metacercariae burden of *Haplorchis* and *Centrocestus* metacercariae in exposed fish were higher than in nonexposed fish, we could not exclude the possibility that some of these metacercariae resulted from exposure and therefore we might have underestimated attack rates. Estimated percentages of infected fish and metacercariae burdens differed between this study and Boerlage et al. (2011); however, the relationship was similar. Sample size in current study was much larger than in Boerlage et al. (2011), which might have resulted in more accurate estimations.

This experimental study shows an inverse relation of percentage of infected fish and trematode burden with fish weight, resulting in higher percentages of infected fish, metacercariae burdens and attack rates to small fish compared to larger fish, but does not provide an absolute threshold fish weight to use in intervention programs. This effect of smaller fish being more easily infected could be more pronounced in seasons when the percentage of infected snails is higher (Karvonen et al., 2006). As cysts remain viable for years and serve as the source of infection to the definitive host (Fried et al., 2004), it is important to prevent fish from getting infected. Intervention methods to improve food safety by targeting fish nurseries, as also recommended by Phan et al. (2010b; 2010c), might reduce the total amount of FZTs in the environment to a large extent. Examples of management practices that can be implemented are e.g. keeping fish fry in cement or composite tanks in filtered water longer than the usual 2 to 7 days (Thien et al., 2009) or changing feed management from a system where fish are fed with manure (Dung et al., 2010) to a system with commercial fish food, a system in which FZT prevalence has been reported to be lower than in a system where fish are fed manure (Thien et al., 2007).

To conclude, it was confirmed that fish weight is an important risk factor for transmission of FZTs and that an inverse relation exists between weight and probability of infection, parasite burden and attack rate. Control measures aiming at reducing transmission to fish fingerlings may reduce the absolute amount of FZTs in the environment, which might lead to a reduced human incidence.

Acknowledgements: The hospitality of Research Institute for Aquaculture No. 1, Dinh Bang, Bac Ninh, Vietnam (RIA1) is very much appreciated by us. Furthermore, we thank Phan Thi Van, vice director of RIA1 and Nguyen Mai Phương and other staff of the Centre for Environment and Disease Monitoring in Aquaculture (CEDMA), part of RIA1, for their assistance and hospitality. Financial support was provided by Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands.

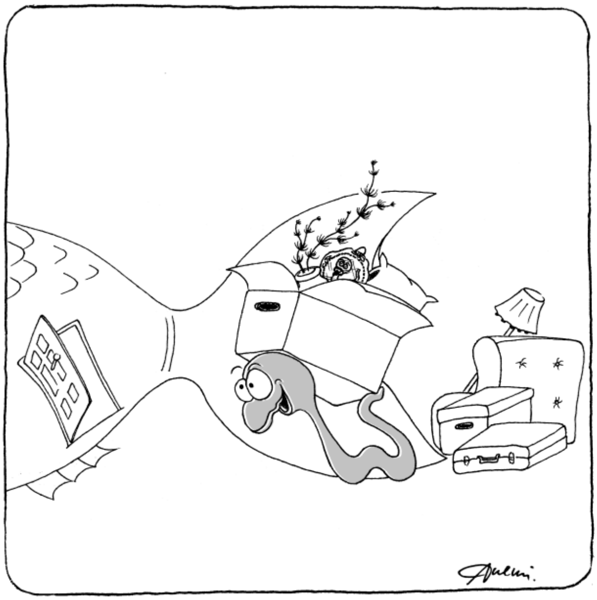
5

Survival of heterophyid metacercariae in common carp (*Cyprinus carpio*)

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands



Parasitology Research (in press)
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Abstract

Heterophyidae are small intestinal trematodes that infect vertebrates worldwide. Common carp (*Cyprinus carpio*) is one of the most preferred freshwater fish species by consumers in Asia, the region where fish-borne trematodes like Heterophyidae are most prevalent. How long Heterophyidae survive in common carp is unknown. The objective of this study was to quantify survival of Heterophyidae in common carp after experimental exposure. Fish of 0.18 g were either used as controls or exposed to 250 heterophyid cercariae for 24 hours. Control fish did not become infected. Percentage infection of exposed fish at 0–2 (*n*=53), >2–10 (*n*=15), >10–20 (*n*=11), and >20–27 (*n*=33) weeks post exposure was 98%, 80%, 100%, and 100% respectively. The number of metacercariae per fish did not significantly decrease (*P*=0.19) during 27 weeks after exposure: exp[3.6200-0.0193·weeks post exposure]. All developed metacercariae were identified as *Haplorchis* spp. It was concluded that Heterophyidae may persist in carps for a long time, implying that harvestable carp are a risk to human health.

Keywords: Heterophyidae, survival, metacercariae, common carp

Introduction

Heterophyidae are small intestinal trematodes that infect birds and mammals worldwide. About 35 species are zoonotic (Chai et al., 2005). The life-cycle of Heterophyidae involves aquatic snail and fish as intermediate hosts and vertebrates, e.g. humans, pigs or chickens, as definitive hosts. Definitive hosts can get infected when they consume raw or undercooked fish (Adams, 2006). Interventions in aquaculture practice could possibly enhance food safety. However, such measures require more insight in the dynamics of heterophyid infections in cultured fish. Therefore, experiments that investigate transmission between Heterophyidae and fish are needed.

Common carp (*Cyprinus carpio*) is one of the most preferred freshwater fish species by consumers in Asia, the region where fish-borne zoonotic trematodes (FZTs) like Heterophyidae are most prevalent (Keiser and Utzinger, 2005; Mohan Day et al., 2005). Prevalences of Heterophyidae in fish can be high, e.g. 43% of common carp was infected in grow-out ponds in northern Vietnam (Chi et al., 2008). Attack rates of Heterophyidae to common carp are known (Boerlage et al., 2011; 2012), but it is unknown how long Heterophyidae survive in common carp. *Haplorchis pumilio* persistsed for at least 9 weeks in cultured tilapia (*Sarotherodon spilurus*) (Sommerville, 1982). *Clonorchis sinensis* metacercariae (*n*=200) persisted more than 26 weeks in the laboratory (Li et al., 2006) and more than 85 weeks post exposure (p.e.) in Chili fish (*Cultriculus eigenmanni*; *n*=27) (Rhee et al., 1985). Mean number of *Centrocestus formosanus* per gill filament declined from 2.2 to 0 in channel catfish (*Ictalurus punctatus; n*=10) at 54 weeks p.e. (Mitchell et al., 2002). Metacercariae count of *Apophallus* spp. and *Nanophyetus salmincola* decreased significantly in coho salmon (*Oncorhynchus kisutch; n*=113) during a 31 week rearing period, however more than 50% of metacercariae of each species persisted. *Neascus* spp. persisted during this period (Ferguson et al., 2010). *Bolbophorus damnificus* persisted in channel catfish *(Ictalurus punctatus*) for at least 30 months (Mitchell et al., 2011).

The objective of this study was to quantify survival time of Heterophyidae in common carp (*C. carpio*) resulting from experimental exposure.

Materials and methods

Common carp fry were purchased from one local fish farmer in Dinh Bang, Bac Ninh, Northern Vietnam in June 2010. Fish were tested free from trematodes using the compressing method (Phan et al., 2010c) before purchase (*n*=30), and one week before start of exposure (*n*=27; weight=0.18±0.09 g). *Melanoides tuberculata s*nails that shed parapleurolophocercous cercariae were obtained from households in Ngia Lac, Nam Dinh province, Vietnam. Exposure doses were prepared similar as in Boerlage et al. (2012). Exposure was performed on 12 days within a period of 6 weeks with respectively 9, 12, 12, 10, 9, 9, 6, 7, 10, 33, 14, and 15 fish per day (*n*=146), and 3 or 4 controls per day (*n*=57). Fish were exposed individually for 24 h to 250 parapleurolophocercous cercariae or were held as controls similar as in Boerlage et al. (2012). Exposed and control fish were kept in one closed recirculation system in net cages; exposed fish of one exposure day were kept in one net cage. Metacercariae were counted after artificial digestion according to Boerlage et al. (2012). A fish was considered infected when at least one metacercariae was detected. Of 146 exposed fish, 34 were excluded because they died during exposure (n = 19), died/were examined < 1 week p.e. (n = 12), or died and were partly eaten by other fish (n = 3). Of the remaining 112, 53 were examined at 1 week p.e., 28 died and were examined at different times during the experiment, and 31 were examined at the last day of the experiment (27 weeks p.e.). For the 57 control fish, 20 were excluded for similar reasons as the exposed fish and the other numbers were respectively 16, 9, and 12. To evaluate the effect of infection with heterophyids on survival times of fish, the Kaplan-Meier survivor functions were estimated for the control and exposed group and compared with the log-rank test (SAS Institute Inc., 2008).

Identifiable metacercariae were of the species *Haplorchis* spp., corresponding to descriptions of Boerlage et al. (2011; 2012). In general, a small amount of the fish harbors most of the parasites (Anderson et al., 1978). Therefore the relation between the number of metacercariae per fish and 'weeks post exposure' (as continuous explanatory variable) was analysed by generalised linear regression using a negative binomial distribution with k as dispersion parameter and a log link function, because it fitted better than using a Poisson distribution (SAS Institute Inc., 2008). If k = 0, the model reduces to the simpler Poisson model; if k > 0 the response variable is over-dispersed; and under-dispersed if k < 0. Corresponding estimate for the number of metacercariae per fish as function of weeks p.e. is expressed as exp[c0+c1 · weeks post exposure]. From this equation, exp[c0] is the initial number of metacercariae after exposure, and 1 − exp[c1] is the survival of metacercariae per week.

Results and discussion

Percentage infected fish examined at 0–2 (n=53), >2–10 (n=15), >10–20 (n=11), and >20–27 weeks (n=33) p.e. was 98%, 80%, 100%, and 100% respectively (Fig. 1). Such a high percentage was also observed by Boerlage et al. (2012) with a similar exposure dose and fish weight. All control fish remained uninfected, which implies that infection in fish was solely due to experimental exposure. Twenty-seven % of fish died during the study. However, mean survival time (±se) of fish was not different between exposed and control fish (respectively 18±1.8 and 15±1.1 days; P=0.95). Therefore, it was assumed that mortality of fish was not a result of experimental infection and that it is valid to use data of fish that died during the experiment in further analysis. Percentage infected fish and number of metacercariae per fish (Fig. 1) was lowest during the first few weeks. This corresponds to observations of Mitchell et al. (2002), who observed the highest mean number of Heterophyidae per channel catfish 7 weeks p.e. The cyst that is formed around metacercariae within the first days p.e. (Komiya, 1966) protects metacercariae during digestion. Possibly, this cyst needs more time to develop before it is strong enough to survive the examination process. Encysting time may be similar to *Ornithodiplostomum ptychocheilus* metacercariae that encysted at 2-4 weeks p.e. in fathead minnows (*Pimephales promelas*) (Sandland and Goater, 2000). Hence, the detectable number of metacercariae in fish examined during the first weeks p.e. may be lower than the real number. According to Umadevi and Madhavi (2006), metacercariae of *Haplorchis pumilio* are fully developed at 2 weeks p.e., which does not correspond to observations that metacercariae appear undeveloped until 3 weeks p.e. (Boerlage et al., 2011). Deleting observations ≤2 weeks or ≤3 weeks p.e. to estimate the number of metacercariae per fish resulted in similar survival estimates.

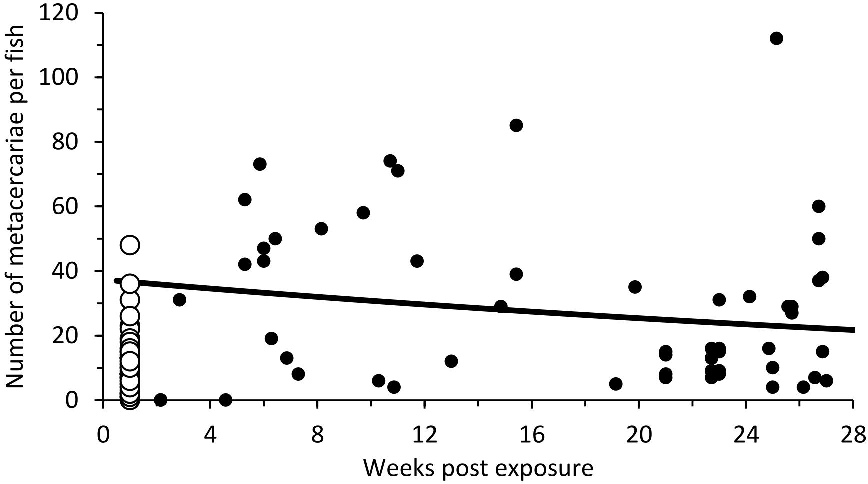

Figure 1. Number of metacercariae per fish. Dots represent observations of fish exposed to 250 cercariae. Only solid dots are observations used to estimate the regression line (n = 59) of the expected number of metacercariae per fish after exposure

The estimated number of metacercariae per fish did not decrease significantly during a period of 27 weeks (P=0.19; k=0.83); exp[3.6200-0.0193·weeks post exposure] (Fig. 1) with 95% confidence limits of the intercept (c0) being 3.0605-4.1795, of the slope (c1) -0.0484-0.0097 and of k 0.5739–1.1899. Amount of over-dispersion was small as a Poisson model showed almost similar results.

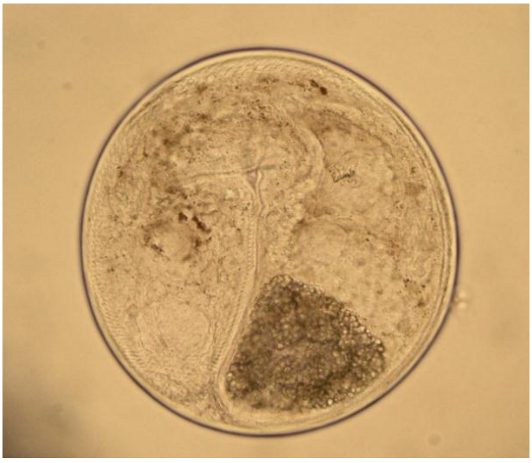

Figure 2. Metacercaria of *Haplorchis* spp.

All cercariae in the exposure dose were of the parapleurolophocercous type. This type is produced by the family Heterophyidae (Schell, 1985). Parapleurolophocercous cercariae cannot be linked to a species based on morphology (Skov et al., 2009), but metacercariae of the family Heterophyidae can (Scholz, et al. 1990). All developed metacercariae were identified as *Haplorchis* spp (Fig. 2). It can be speculated that these cercariae were of the species *Haplorchis pumilio*, because this species occurs in 62% of fish from ponds where the snails were obtained, whereas other trematodes species occur in 2% or less of the sampled fish (Phan et al., 2010c). Because the presence of other heterophyid species cannot be excluded, we refer to Heterophyidae in this study.

In conclusion, prevalence and abundance of Heterophyidae in common carp does not decrease during a period of 27 weeks. As common carp hardly acquire new infections after weighing 40 grams (Boerlage et al., 2011; 2012), and infections seem to persist, carps might still have about maximum heterophyid levels at harvesting and are therefore a risk for public health.

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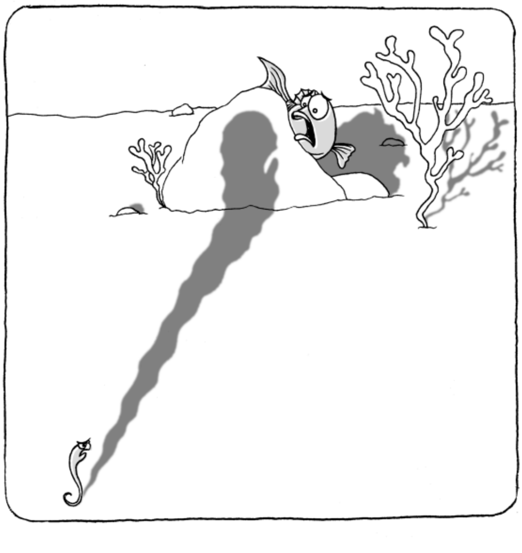
6

Transmission of fish-borne trematodes (*Heterophyidae*) to common carp (*Cyprinus carpio*) is independent on fish and trematode density

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands



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Abstract

Fish-borne zoonotic trematodes (FZTs) can cause major human health problems. The aim of this study was to quantify the transmission of parapleurolophocercous cercariae to common carp (*Cyprinus carpio*) and to study the effect of the density of cercariae and the density of fish on transmission with respect to the volume of water and surface area of the bottom. Fish were kept individually either as controls (*n*=91) or were exposed to 250 cercariae in tubes with a volume of 25, 50, 100, 250 or 500 ml water (*n*=190) with a surface area of 4, 12, 21, 30, or 49 cm^2 (*n*=195). The dose to which the fish were exposed was kept constant. Infection occurred in 94-100% of fish, with a mean of 15-18 metacercariae per fish and the proportion of FZTs established at 0.06-0.07 metacercariae per cercariae per fish. Neither the prevalence of infection with FZTs nor the number of metacercariae per fish or the proportion of FZTs established were significantly associated with differences in the density of cercariae or the density of fish per ml water or per cm^2 surface area. Thus, it was concluded that the transmission of cercariae to fish is independent of density.

Keywords: transmission, common carp, fish-borne zoonotic trematode, density of cercariae, density of fish

Introduction

Fish-borne zoonotic trematodes (FZTs) are commonly reported worldwide, and more than 70 species are known to be zoonotic (WHO, 2011). The lifecycle of FZTs involves a primary snail host, secondary fish hosts and final hosts, such as humans, pigs or fish-eating birds (Chai *et al*., 2005). Humans and other final hosts can become infected when they eat raw or undercooked infected fish (Chai et al., 2005; Phan et al., 2011). In addition, certain aquaculture management practices, such as using excreta from final hosts as manure in ponds, can enhance the persistence of FZTs (Thien et al., 2007; Dung et al., 2010; Lima dos Santos and Howgate, 2011). Currently, the primary intervention strategy for human infections is prophylaxis (WHO, 2011). However, additional intervention methods that can be applied in aquaculture, for example reducing transmission to fish fingerlings (Boerlage et al., 2012) by keeping fish fry in filtered water in cement tanks longer than the usual 2 to 7 days (Thien et al., 2007), require further research to identify the mechanisms involved in the transmission of FZTs to fish.

Transmission of FZTs to fish occurs via cercariae that emerge from snails (Lo and Lee, 1996a; Chai et al., 2005). In general, transmission can be categorized as density-dependent or frequency-dependent (e.g. Keeling and Rohani, 2008; Vynnycky and White, 2010). In density-dependent transmission, contacts depend on the size of a designated space (e.g. area or volume) in which contact can take place (Begon et al*.,* 2002). For cercariae-to-fish transmission, the designated space is a body of water, *for example* a fish tank, pond or lake. In frequency-dependent transmission, contacts are independent of the size of a designated space. However, the term frequency-dependent may be misleading when comparing transmission between the two models because 'frequency-dependent' is always used when extrapolating between situations with the same density (De Jong et al., 1995; Bouma et al., 1995). Therefore, in this study, we refer to 'density-dependent' and 'density-independent' transmission. Transmission of FZTs to fish in aquaculture takes place in a more-or-less closed designated area where the dimensions of the water depth and surface area of the base of the fish enclosure can be regulated, as well as the number of fish. A different number of fish per designated area may have an effect on the number of trematodes per fish, which may be relevant to human health.

It is therefore important to know whether transmission occurs in a density-dependent fashion.

To determine whether transmission is density-dependent or -independent, the size of a designated space can be varied using similar numbers of cercariae and fish. As both cercariae and fish move in 3 dimensions in water bodies, the size of the designated space, i.e. the water body, can be expressed as a volume. However, cercariae tend to accumulate near the surface of the water (Feiler and Haas, 1988), in midwater (Haas et al*.*, 1990), or at the bottom (McCarthy et al*.*, 2002). Furthermore, fish can be distributed unevenly in the water (Gibson, 1972). For example, the common carp (*C. carpio*) spends about half of its time at the bottom (Rahman et al., 2008). Therefore, cercariae and/or fish may not be homogeneously distributed in different depths, and the surface area of the bottom might be a better measure than the volume to describe the designated space occupied by fish and cercariae.

The transmission of cercariae to fish has been quantified at either a constant dose of cercariae/volume or a constant area/volume. In the latter case, the density of cercariae increases with increasing doses of cercariae, and thus in both cases, the effect of the dose and the density of cercariae cannot be disentangled. However, the intensity of infection or the number of metacercariae per fish depends on the dose to which fish were exposed in a constant volume of water for the transmission of the non-FZT *Transversotrema patialense* cercariae to zebra fish (*Brachydanio rerio*), the non-FZT *Diplostomum spathaceum* cercariae to rainbow trout (*Oncorhynchus mykiss*), and the FZT parapleurolophocercous cercariae (*Heterophyidae*) to common carp (*C. carpio*) (Anderson et al., 1978; Karvonen et al., 2003; Boerlage et al., 2011). In addition, for the non-FZT *D. spathaceum* cercariae, Karvonen et al. (2003) observed no effect on transmission due to the density of cercariae in a constant volume of water and a reverse effect on transmission due to the dose to which fish were exposed at a constant density of cercariae. Thus, these authors concluded that transmission was independent of density.

In designing our experiment, we believed that additional insight into the effect of density on transmission would be gained by disentangling the effect of the dose of cercariae and the density of cercariae. Thus, our approach was different than studies on density- or frequency-dependent transmission because density was evaluated per volume of water and also per surface area of the bottom by maintaining the dose of cercariae constant. Therefore, the objective of our study was to quantify the effect of density with respect to the volume of water and the surface area of the bottom on the transmission of FZT cercariae to common carp (*C. carpio*).

Materials and methods

Experimental infection of fish

The larval stage of FZTs that emerge from snails, i.e. cercariae, was used to infect the fish. *Melanoides tuberculata* snails from which parapleurolophocercous cercariae emerged were obtained from Ngia Lac, Nam Dinh Province, Vietnam and brought to the Research Institute for Aquaculture No. 1, Bac Ninh province, Vietnam, where the experiments were carried out. Cercariae were counted as they were drawn into a pipette. Doses of cercariae and volumes of water were prepared according to Boerlage et al. (2011). Common carp (*Cyprinus carpio*) of unknown exposure history and age were obtained from one local fish farmer. After exposure, the fish were artificially digested according to Boerlage et al. (2011), after which the numbers of the consecutive larval stage of FZTs in fish, i.e. metacercariae, were counted. Counted metacercariae were of the morphologically distinct groups *Haplorchis, Centrocestus* and 'undeveloped'. *Haplorchis* and *Centrocestus* forms were considered present prior to experimental exposure. Undeveloped metacercariae were considered to be the result of experimental exposure, and these differed from *Haplorchis* and *Centrocestus* because of their formless, motionless appearance in which no organs could be distinguished. This method of distinguishing metacercariae that were 'already present' from those that 'originated from experimental exposure' has been described and discussed in detail by Boerlage et al. (2011) and was considered justified because there was a dose-response relationship for 'undeveloped' metacercariae that did not exist for *Haplorchis* and *Centrocestus.* Undeveloped metacercariae were most likely members of the genus *Haplorchidae* because the prevalence of species belonging to this genus was highest in the area where the snails were collected (Dung et al., 2007; Lan Anh et al., 2009b; Phan et al., 2010c).

For sample size estimation, a pilot study was performed in 2009 to estimate the effects of the dose of cercariae and the volume of water during exposure on the prevalence of infection with FZTs, the number of metacercariae per fish and the proportion of FZTs established per common carp (1.07±0.27 g). Sample sizes were calculated using WIN EPISCOPE 2.0 (Thrusfield et al., 2001) at 95% confidence (one tailed) and 80% power. Sample size calculations to detect a difference between 80 and 100% prevalence resulted in 26 fish per group. To detect a difference between 1 and 8 metacercariae per fish with an SD of 6, 12 fish per group were needed. For differences in the proportion of FZTs established per fish between 0.1 and 0.15 and an SD of 0.07, 27 fish per group were needed. Based on these calculations, it was decided to use at least 30 fish per group.

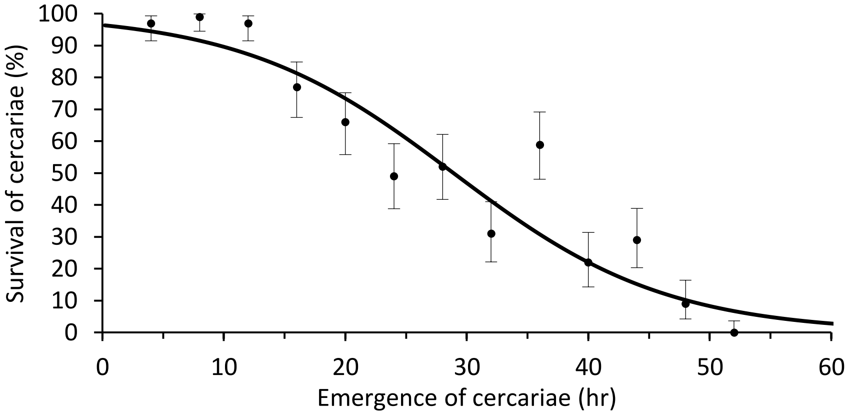

Figure 1. The observed (•), with exact 95% confidence interval (⊥), and expected (—, 100/(1 + exp[-3.3947 + 0.1147•hours post cercariae emerging)]) proportion of surviving cercariae (%).

The survival of cercariae that emerged from the snails was determined. Snails were obtained 3 months prior to the exposure of fish to cercariae. Snails were allowed to shed cercariae for 15 minutes. Subsequently, 1,300 cercariae were divided between glass cups containing 3 ml water with 10 cercariae per cup. Room temperature was between 29.2 and 31.3 °C. Cercariae were defined as ‘dead’ when they remained immobile after being stimulated by a needle. The survival of cercariae was recorded for 100 cercariae (10 cups) every 4 hours until 52 hours post-emergence of the cercariae (13 sampling times). Survival was recorded only once per sample of cercariae because stimulation during the recordings may have affected cercariae survival. The survival percentage decreased significantly with time ($P<0.01$) (Fig. 1). Given a 4 hour time for dose preparation, it was decided to let the emergence time of the cercariae be 8 hours, so that fish were exposed to cercariae of a maximum age of 12 hours and that the estimated proportion of living cercariae was approximately 85%.

Common carp (0.34±0.17 g) were purchased 20 days prior to exposure in October 2010. The fish were kept in one tank containing 80 L of well water until exposure. The water temperature was between 26 and 29°C.

Fish were either kept as controls (n=91) or exposed to 250 cercariae to estimate the effect of the density of cercariae and the density of fish with respect to the volume of water (n = 190) and the surface area of the bottom (n=195) (see Table 1). In total, nine treatment groups were present. Tubes with respectively 4, 12, 21, 30, or 49 cm^2 were filled with 100 ml water, and tubes with respectively 25, 50, 100, 250 or 500 ml water had a bottom surface area of 21 cm^2.

Table 1. Experimental design to demonstrate the effect of the volume of water, surface area of the bottom and height of the water in tubes on carp that were exposed to 250 *Heterophyidae*.

Volume of water ml	Surface of bottom cm^2	Height of water mm	Sample size *n*
25	21	12	33
50	21	24	32
100	21	47	61[a]
250	21	118	32
500	21	235	32
			n=190
100	4	241	33
100	12	84	33
100	21	47	61[a]
100	30	33	32
100	49	20	36
			n=195

[a]: the same group of fish

The exposures were performed on 10 separate days within a period of 36 days with respectively 45, 29, 25, 20, 15, 31, 48, 36, 45, and 30 fish exposed. On each exposure day, all nine treatment groups were represented. Per treatment group per exposure day, one control fish (no exposure to cercariae) was used, resulting in nine control fish examined on each exposure day. However, on the first exposure day, ten control fish were present. Two fish that died between exposure and digestion were stored at 5°C until digestion.

Data analysis

As data obtained on one exposure day may not be independent, the exposure day was included as a random variable using an exchangeable correlation structure.

The presence or absence of infection was analyzed by logistic regression using a binomial distribution and logit link function (PROC LOGISTIC, SAS Inst. Inc., 2008). A fish was considered infected when at least one metacercariae was found, and the results were expressed as the prevalence of infection (%). In cases of 0 or 100% infection, random effects could not be included, and an exact test then was used.The number of metacercariae per fish was analyzed using generalized linear models with a Poisson distribution and a log link function (PROC GENMOD, SAS Inst. Inc., 2008). The mean numbers of within-groups were adjusted for other effects in the model and expressed as least square means (lsmeans) ± standard error of the mean (S.E.M.) per treatment (SAS Inst. Inc., 2008). When 0 metacercariae were present, lsmeans could not be estimated.

The proportion of FZTs established, i.e. the number of new metacercariae per cercariae exposed to fish, was analyzed using generalized linear models with a Gaussian distribution (PROC MIXED, SAS Inst. Inc., 2008). Means resulting from the model were expressed as the lsmeans ± S.E.M. per treatment.

Results

In exposed and control fish, the prevalence of infection with *Haplorchis* metacercariae was respectively 0.3% and 1.1% and that with *Centrocestus* was respectively 46.6% and 40.7%, with no significant difference between the two (respectively P=0.38 and P=0.31) (Table 2). For exposed and control fish, the mean number of metacercariae per fish was between 0 and 1 for both *Haplorchis* and *Centrocestus* (respectively P=0.38 and P<0.01). Undeveloped metacercariae were absent in control fish but present in 98% of exposed fish ($P<0.0001$), with a mean number of 15 metacercariae per fish ($P<0.0001$).

Table 2. The effect of the dose of cercariae on the prevalence (%) of carp infected with metacercariae (mean ± S.E.M.) of the *Haplorchis*, *Centrocestus* and undeveloped forms.

Dose of cercariae	Sample size *n*	*Haplorchis*		*Centrocestus*		Undeveloped	
		%	Mean±SEM	%	Mean±SEM	%	Mean±SEM
0	91	1.1	0.01±0.01	40.7	0.52±0.08	0	0.00±0.00
250	324	0.3	0.00±0.00	46.6	0.77±0.05	98.2	14.56±0.21
P-value		0.3828	0.3842	0.3127	0.0076	<0.0001	<0.0001

No differences were observed regarding the prevalence of infection, the number of metacercariae per fish or the proportion of FZTs established per fish for fish exposed to 250 cercariae in different densities per volume of water (Table 3). The prevalence of infection due to exposure varied between 94 and 100% ($P=0.42$). The mean number of metacercariae varied between 16 and 18 metacercariae per fish ($P=0.27$), and the exposure day explained 52% of the unexplained variance. The proportion of established FZTs varied between 0.06 and 0.07 metacercariae per cercariae per fish ($P=0.51$), and the exposure day explained 67% of the unexplained variance. Density of cercariae did not explain the variation in the proportion of established FZTs ($0.06337+(9.31 \bullet 10^{-4}$xnumber of cercariae per ml) ($P=0.14$; R^2 below 0.1%).

Table 3. The prevalence (%) of carp infected with metacercariae of the *Haplorchis*, *Centrocestus* and undeveloped forms and the proportion of established undeveloped metacercariae (mean ± S.E.M.) relative to the volume of water in tubes during exposure.

Volume of water (ml)	Sample size *n*	*Haplorchis*[a] %	*Centrocestus* %	*Centrocestus* Mean±SEM	Undeveloped %	Undeveloped Mean±SEM	Undeveloped Mean established ±SEM
500	32	0	59	1.2±0.3	100	16.3±2.5	0.065±0.014
250	32	0	47	0.7±0.2	100	15.6±2.9	0.062±0.014
100	195	1	44	0.7±0.1	98	16.3±3.2	0.065±0.013
50	32	0	41	0.5±0.1	100	18.2±3.1	0.073±0.014
25	33	0	58	0.9±0.1	94	17.9±3.5	0.071±0.014
P-value:		1.0000	0.3011	0.0764	0.4222	0.2653	0.5080

[a]: the mean number of metacercariae was not estimable because the number of infected fish (n = 1) was too low.

No differences were observed regarding the prevalence of infection with FZTs, the number of metacercariae per fish or the proportion of FZTs established per fish for fish exposed to 250 cercariae at different densities per surface area of the bottom (Table 4). The prevalence of infection with FZTs due to exposure varied between 97 and 100% (P=0.75). The number of metacercariae varied between 15 and 17 metacercariae per fish (P=0.42), and the exposure day explained 52% of the unexplained variance. The proportion of established FZTs varied between 0.06 and 0.07 metacercariae per cercariae per fish (P=0.46), and the exposure day explained 66% of the unexplained variance. The density of cercariae did not explain the variation in the proportion of established FZTs ($0.06734-(6.35 \bullet 10^{-5}$xnumber of cercariae per cm^2) (P=0.55; R^2 below 0.1%).

Table 4. The prevalence (%) of carp infected with metacercariae of the *Haplorchis*, *Centrocestus* and undeveloped forms and the proportion of established undeveloped metacercariae (mean ± S.E.M.) relative to the surface area of the bottom in tubes during exposure.

Surface of bottom (cm^2)	Sample size n	*Haplorchis*[a] %	*Centrocestus* %	*Centrocestus* Mean±SEM	Undeveloped %	Undeveloped Mean±SEM	Undeveloped Mean established ±SEM
49	36	0	33	0.6±0.2	97	14.7±3.0	0.060±0.014
30	32	3	53	0.9±0.2	97	16.0±3.2	0.064±0.014
21	190	0	50	0.8±0.1	98	17.1±3.0	0.068±0.013
12	33	0	42	0.7±0.2	100	16.8±3.4	0.067±0.014
4	33	0	39	0.6±0.2	100	15.4±3.4	0.062±0.014
P-value		0.0988	0.2910	0.4472	0.7475	0.4200	0.4637

[a]: the mean number of metacercariae was not estimable because the number of infected fish (n = 1) was too low.

Discussion

Our objective was to quantify the effect of density with respect to the volume of water as well as the surface area of the bottom on the prevalence of infection with FZTs, the number of metacercariae per fish and the proportion of FZTs established per fish in common carp (*C. carpio*). In contrast to other studies, the dose of cercariae per fish was kept constant in all treatments in our study. Therefore, the density of cercariae and the density of fish per volume of water or surface area of the bottom were 100% co-linear, and 'density' related to both the fish and cercariae. Extrapolation of the results into a situation where the dose of cercariae per fish varies, as in Karvonen et al. (2003), may result in different proportions of FZTs established per fish.

Of all the fish exposed, 98% were infected. No differences were observed between the number of metacercariae per fish and the proportion of FZTs established per fish at different densities using a sample size that would have detected differences between treatments, as observed in the pilot experiment. It was therefore concluded that there was no effect of density on transmission. The outcomes for the number of metacercariae per fish were comparable to the estimates of Boerlage et al*.* (2011, 2012) for fish of a similar size that were exposed to the same dose of cercariae. However, our results may have been slightly underestimated because our calculations were performed based on a dose of 250 cercariae, whereas the survival curve indicated that on average, 85% of cercariae were expected to be alive. As this was similar for all treatments, it was assumed that this did not have a large influence on the observed relation between density and transmission. The exposure day explained more than half of the unexplained variance, which implies that differences between exposure days had a large effect on transmission. One potential factor that may affect transmission and that differed between exposure days was the temperature. Although the water temperature during exposure days differed by a maximum of only 4°C, this could have caused the effect due to the exposure day. This effect was also observed by Stables and Chappell (1986), as they documented that the difference between no transmission and maximum transmission of *Diplostomum spathaceum* to rainbow trout (*Salmo gairdneri*) was caused by an approximate 7.5°C difference in the water temperature.

However, it remains unclear how these findings should be extrapolated to large water bodies. The densities used in our experiment are in line with those used in fish ponds in aquaculture. For example, in a 0.58 m deep fish pond of 652 m^2 (Nhan et al., 2006) with 4,000 snails per m^2 (Kittivorachate and Yangyuen 2004), and 13% of snails with an average of 689 *Haplorchis pumilio* cercariae emerging per day (Lo and Lee, 1996b; Dung et al., 2010), the density of cercariae is 600 per L and 350,000 per m^2 (250 cercariae per 417 ml and per 7 cm^2 on the scale of our experiment). When extrapolated, the densities of fish in our experiment were between 200 and 2500 fish per m^2 and covered the range of the densities advised for fish ponds, i.e. 100-400 common carp fry per m^2 (Peteri, 2012). The lack of an effect on transmission due to density implies that the expected proportion of cercariae established per fish in a larger designated space, for example a fish pond, may be similar to those observed during the experiment. However, the number of metacercariae per fish in fish populations is generally over-dispersed (Anderson and Gordon, 1982), which may be caused by either heterogeneity in the susceptibility of fish to cercariae or generated by factors such as differences in fish behaviours (Anderson et al., 1978; Höglund, 1995). Therefore, non-statistical extrapolation of results from situations with individual fish, as in our experiment, to situations with a larger fish population may not be straightforward. Furthermore, as most cercariae actively search for their host (Haas, 2003), one can speculate that at very low densities, the few cercariae present might not find the few fish in a large fish pond. This corresponds to the expectation that at very low densities, the relationship between density and contact rate is different than at average densities (Diekmann et al., 1995).

To our knowledge, this was the first study to quantify the transmission of FZT cercariae to fish with respect to density by exposing fish to a constant dose of cercariae. It was concluded that transmission was not affected by changes in the density of either cercariae or fish.

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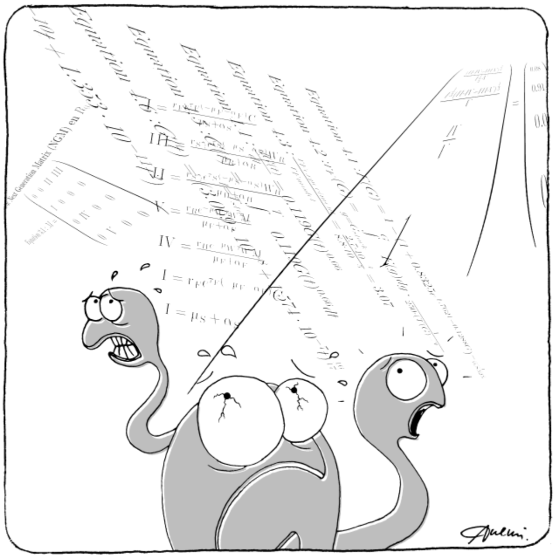
7

Effect of control strategies on the persistence of fish-borne zoonotic trematodes: a modelling approach

Annette S. Boerlage[a], Elisabeth A.M. Graat[a], Johan A. Verreth[b], Mart C.M. de Jong[a]

[a] Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands

[b] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, Wageningen, The Netherlands



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Abstract

Fish-borne Zoonotic Trematodes (FZTs) are a risk to human health and need to be controlled. A mathematical model was developed to give insight into how and to what extent control strategies change the dynamics of FZTs on integrated agriculture-aquaculture farms. The reproduction ratio R evaluates the effects of control strategies. R>1 implies that the infection may persist, whereas R<1 implies that the infection certainly cannot persist. In the absence of control strategies (default), R=1.92. After implementing control strategies either (i) R and percentages infected hosts in the equilibrium did not change and FZTs persisted (ii) R became smaller, but not below 1, the new equilibrium had lower proportions of infected hosts, and FZTs persisted, or (iii) R became smaller than 1, and all hosts were FZT-free in the new equilibrium. Single chemotherapy of humans, reservoir hosts or both did not change R. Continuous chemotherapeutic treatment reduced R but not below 1 when treating only humans (R=1.30) or only reservoir hosts (R=1.69). A combination could result in R<1, e.g. treating all humans and >54% of reservoir hosts. Snail control could result in R<1 with a decrease in density or increase in mortality of snails. This will occur when either transmission to snails or to fish is <14% of its default value. Stocking fish at >25 g as compared to 0.5 g that is usual in aquaculture practice, or at >14 g in combination with treating all humans, led to R<1. Advantage of using R for evaluating control strategies is that it provides insight into control success or failure even if it would take several decennia to observe this effect in the field.

Keywords: fish, trematodes, aquaculture, zoonosis, control strategies, modelling

Introduction

Fish-borne zoonotic trematodes (FZTs) occur worldwide and are endemic in most countries in Asia. More than 20 million people are infected with FZTs in China, Thailand, Lao People's Democratic Republic and South Korea alone (WHO, 2004). Best known FZTs are the liver flukes *Clonorchis sinensis, Opisthorchis viverrini*, and *Opisthorchis felineus*. They can lead to cholangitis, choledocholithiasis, pancreatitis and cholangiocarcinoma in humans (Chai et al., 2005; Rim, 2005). Another group of FZTs are the intestinal flukes, like *Haplorchis pumilio* and *Metagonimus yokogawai*. In most cases, these produce mild intestinal disturbances in humans (Chai et al., 2005; Velasquez, 1982; Yu and Mott., 1994).

The life-cycle of FZTs involves aquatic snails as primary hosts, fish as secondary hosts and vertebrates e.g. humans, pigs, cats or fish eating birds as definitive hosts (Lima dos Santos and Howgate, 2011). In short, infected definitive hosts excrete FZT eggs with their feces which subsequently may be eaten by snails. Multiple larval stages occur in snails leading to cercariae. Cercariae are shed into the water, penetrate the skin and the underlying muscle of fish and encyst in muscle tissue of fish where they develop into metacercariae. When definitive hosts eat raw or undercooked fish, metacercariae are released and develop into adult worms that excrete eggs (Komiya, 1966).

To avoid human cases, the most obvious control strategy against FZTs is eliminating transmission to humans, e.g. by preventing humans from eating raw or improperly cooked fish. Health education efforts aimed at changing such traditional habits have not been very successful (WHO, 2004; 2011). In addition to providing information about the risks of eating raw fish, preventive chemotherapeutic treatment of humans is currently the main control strategy against Opisthorchis and Clonorchis infections (WHO, 2011). The effect of treating humans may be limited because the life-cycle can continue through other fish-eating vertebrates. Therefore humans may get re-infected once treatment is stopped (Chai et al., 2005).

The occurrence of FZTs is associated with aquaculture (Lima dos Santos and Howgate, 2011). On the one hand integrated agriculture-aquaculture (IAA) systems improve the livelihoods of small scale farmers (FAO, 2001; Prein, 2002). On the other hand they may enhance transmission of FZTs, because snails, fish, humans and other vertebrates can be present on a single farm and their manure is often used to fertilize fish ponds (Dung et al., 2010; Sapkota et al., 2008).

Long-term epidemiological studies are expensive and time consuming. Also, if conditions change during the study these may affect the results of the study, and loss to follow-up is very likely to occur. As a consequence, the effect of control strategies in the long run (after several decennia) remains unknown. One powerful tool that can be used to obtain more insight into the effect of control strategies on the dynamics of FZTs in the different hosts over time is mathematical modelling (De Jong, 1995). Transmission of FZTs between hosts can be modelled and the effects of control strategies can be compared using the reproduction ratio (R) in the situation with and without control strategies. The R is the average number of new cases caused by 1 typical infectious individual in a fully susceptible population (Diekmann et al., 1990). An R>1 implies that the infection could possibly persist in the host populations. An R<1 implies that the infection certainly cannot persist, as the special conditions that lead to exceptions to that general rule do not apply here (Greenhalgh et al., 2000).

Objective of this study was to give insight into how and to what extent control strategies will change the dynamics of FZTs on IAA farms.

Materials and methods

Model description

The life-cycle of FZTs on IAA farms (Fig. 1) is modeled using Mathematica® 8 (Wolfram, 2011). The model is a deterministic compartmental SI model (Keeling and Rohani, 2008) with the proportion of infected hosts (I) and susceptible hosts (1-I) as variables. Ordinary differential equations (ODEs) describe changes in the proportions infected hosts per day on an IAA farm. The host types are: snails, fish, humans and reservoir hosts (definitive hosts other than humans). The proportions infected hosts increase in time when susceptible hosts become infected and decrease if hosts die or become FZT-free due to control strategies. Snails and fish are assumed to remain infected once infected, as mortality rates of trematodes in snails and fish are low compared to mortality rates of snails and fish (Attwood and Chou, 1978; Boerlage et al., 2013*c*; Chen et al., 1994; Fried et al., 2004; Keiser and

Utzinger, 2005; Wright, 1971). For definitive hosts, only the host mortality rate of humans is lower than the mortality rates of FZTs (Choi et al., 2004) and thus humans may become disease free.

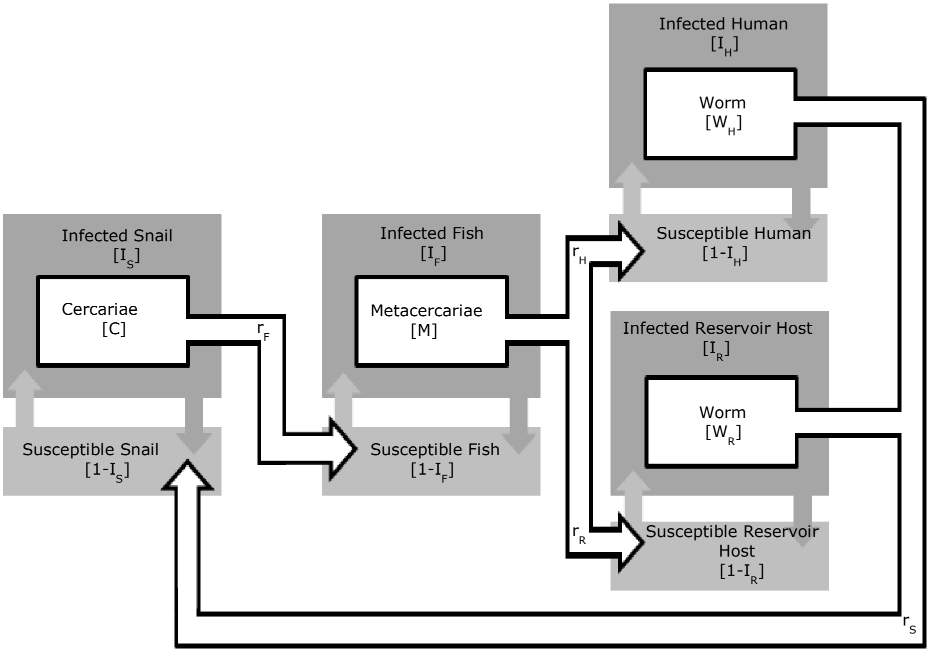

Figure 1. Flowchart of the life-cycle model of FZTs. The host population that consists of infected (I) and susceptible (1-I) hosts are in gray, parasite stages are in white and transmission rates of FZTs to hosts (resp. r_S, r_F, r_H, r_R for snail, fish, human and reservoir host) are indicated in white arrows.

Density of snails and fish (number per m^2), and total number of humans and reservoir hosts are assumed to be constant. Therefore, we assume that birth rate equals total mortality (natural and parasite dependent) of hosts. Values for all parameters are obtained from literature and used as default values (Table 1). See Appendix A for derivations of the ODEs.

Values of the transmission rates are estimated based on observational studies describing infections of FZTs in hosts on IAA farming systems in Nam Dinh province, Vietnam. In this area, about 13% of *Melanoides tuberculata* snails (Boerlage et al., 2013*a*; Dung et al., 2010), 65% of cultured fish in ponds (Phan et al., 2010b), 50% of humans (Dung et al., 2007; Phan et al., 2011) and 61% of reservoir hosts (Lan Anh et al., 2009a; average of dogs, cats, and pigs) were infected with FZTs. These percentages are assumed to be at the stable endemic equilibrium, and are used to estimate the transmission rates to snails, fish, humans and reservoir hosts. These transmission rates are used as default values (Table 1). Relative reduction proportions (p) are used to model how control measures change the transmission rates, i.e. at default transmission rate p=1, and for e.g. 50% reduction in transmission p=0.5. See Appendix B for estimations of the transmission rates and further explanation.

The Next Generation Matrix (NGM) is derived from the ODEs (Diekmann et al., 2010; see Appendix C for explanation and estimations). In brief, the NGM enables to calculate the next generation of infected individuals for each infected host type (snail, fish, human and reservoir host) from the infected individuals for each infected host type in the current generation. From the NGM, the dominant eigenvalue (=R) and its corresponding eigenvector are derived. The eigenvector is the typical infected individual according to the definition of R. The NGM, R and eigenvector provide insights into if, how and to which extent the different parameters affect the dynamics of FZTs in hosts. A sensitivity analysis of parameter values shows if and how a change in value of 1 parameter affects R if all other values remain at their default value, and whether this can lead to R<1.

Control strategies

The model with all parameters at the default values (Table 1) is referred to as the default situation (i.e. without control). Control strategies are simulated by changing the value of 1 or more parameter(s) while values of other parameters remain at their default value. The value of R for the new parameter combination is calculated.

1. Single chemotherapy of either humans, or reservoir hosts or both. After chemotherapy, hosts become FZT-free (Fried et al., 2004) and thus susceptible. In the model, this is incorporated by changing the percentage of infected humans, reservoir hosts or both to 0 (Table 3) at time t = 0.

2. Continuous chemotherapy of either humans, or reservoir hosts or both. Contrary to the single chemotherapy, percentages for the treated hosts are kept 0 at all times by starting with 0 infected hosts and

additionally changing transmission rates to hosts to 0, because hosts cannot get re-infected (Table 3).

3. Control of snails, e.g. by poly-culture with snail-eating fish or applying molluscicides, can be a potential control strategy (Ben-Ami and Heller, 2001; Hoffman, 1970; Molyneux, 2006; Venable et al., 2000; WHO, 1995). This is modelled by changing the parameters density and mortality of snails.

4. Delayed stocking of fish. Before being stocked in a pond, fish fry are kept under FZT-free conditions (e.g. concrete fish tanks with filtered water), usually for 2 – 7 days (Thien et al., 2009). The transmission rate to fish decreases with increasing fish weight (Boerlage et al., 2011; 2012). If stocking of fish in ponds is delayed, fish weigh more when first exposed to FZTs. This will result in a lower overall transmission rate to fish, and might lead to R < 1. Fish weight at stocking corresponding to R < 1 is calculated either as a single control strategy or in combination with continuous chemotherapy of humans.

Results

Estimated transmission rates to snails (r_S), fish (r_F), humans (r_H), and reservoir hosts (r_R) are resp. 1.8 x 10^{-7}, 8.8 x 10^{-5}, 1.0 x 10^{-8}, and 7.2 x 10^{-6} hosts per FZT per day (Appendix B). In the default situation, thus without control strategies, each infected snail infects on average 22 fish, each fish 0.03 reservoir hosts and 0.00004 humans, each reservoir host 4 snails, and each human 5914 snails (Table 2). In the default situation, R = 1.92 (for explanation of symbols see Table 1, for derivation see Appendix C), implying that FZTs can persist (see also Fig. 2). The distribution of infected hosts at the stable equilibrium is: 7.9% of snails, 90.8% of fish, 0.002% of humans and 1.2% of reservoir hosts.
The equation for R is:

$$R = 2.42643\left(\frac{p_S\, p_F(0.229885\, p_H\, N_H\, W_H + 0.359564\, p_R\, N_R\, W_R)}{(0.5\, N_H\, W_H + 0.61\, N_R\, W_R)}\right)^{\frac{1}{3}}$$

Table 1A. Parameters used in the model with symbols, default values and values at which R < 1 for each type of host; snail.

Parameter	Symbol	Default value	Species	Literature source	Value R < 1
Natural mortality of snails	μ_S	6.1×10^{-4} per day	*M. tuberculata*	Elkarmi and Ismail, 2007	NP[a]
Parasite dependent mortality of snails	α_S	0 per day	*M. tuberculata* and parapleurolophocercous cercariae		NP
Average number of cercariae shed per infected snail per day	C	163 cercariae per day[c]	*M. tuberculata* and parapleurolophocercous cercariae	Bae et al., 1983	NA[b]
Development time from ingested egg to cercaria in snails	τ_S	80 days	*C. sinensis*	Reviewed by Chen et al., 1994	NA
Average number of eggs excreted per worm per day	γ	3160 eggs per worm per day[c]	*C. sinensis*	Kaewkes, 2003; Wykoff, 1959	NA
Pond size	A	100 m^2		Luu 2001	NA
Density of snails	N_S	100 snails per m^2	*M. tuberculata*	Dudgeon, 1986; Genner et al., 2008	NA
Relative reduction proportion of transmission to snails	p_S	1			< 0.14
Transmission to snails	r_S	1.8×10^{-7} snails per egg per day		(Appendix 2)	$< 2.5 \times 10^{-8}$

[a] NP: R cannot become lower than 1 within biologically plausible values of this parameter.

[b] NA: not applicable as parameter is not part of equation for R.

[c]: Cercariae (Lo and Lee 1996b; Boerlage et al., 2013b) and eggs were assumed to be infectious for 1 day.

Table 1B. Parameters used in the model with symbols, default values and values at which R < 1 for each type of host; fish.

Parameter	Symbol	Default value	Species	Literature source	Value R < 1
Mortality of fish (in aquaculture)	μ_F	4.7×10^{-3} per day	Fish[e]	Pekar et al., 2002	NA
Parasite dependent mortality of fish	α_F	0 per day			NA
Average number of metacercariae per infected fish	M	17 metacercariae	*Haplorchis* in common carp	Boerlage et al., 2012	NA
Development time of metacercariae in fish	τ_F	15 days	*H. pumilio* in freshwater fish	Umadevi and Madhavi, 2006	NA
Density of fish	N_F	20 fish per m^2	Fish*	Luu, 2001	NA
Relative reduction proportion of transmission to fish	p_F	1			< 0.14
- Transmission to fish[d]	r_F	8.8×10^{-5} fish per cercaria per day		(Appendix 2)	$< 1.3 \times 10^{-5}$
- Stocking weight of fish[d]	G_{stock}	0.5 g	Common carp	WRC, 2010	25
- Harvesting weight of fish[d]	$G_{harvest}$	800 g	Common carp	Hepher, 1988	NP
- Grow-out period of fish (between stocking and harvesting)[d]	$T_{harvest} - T_{stock}$	220 days	Common carp	Nhan et al., 2006	166

[d]: in integrated agriculture-aquaculture (IAA) farming systems.

Table 1C. Parameters used in the model with symbols, default values and values at which R < 1 for each type of host; human and reservoir host.

Parameter	Symbol	Default value	Species	Literature source	Value R < 1
Natural mortality of humans	μ_H	$3.8\text{x}10^{-5}$ per day	Humans	CIA, 2012	NA
Parasite dependent mortality of humans	α_H	0 per day		Chen et al., 1994	NA
Average number of worms per infected human	W_H	416 worms	*H. pumilio*	Dung et al., 2007	NP
Natural mortality worm in definitive host	μ_W	$1.2\text{x}10^{-4}$ per day	*C. sinensis*	Choi et al., 2004	NA
Development time of worm	τ_W	26 days	*C. sinensis*	Komiya, 1966	NA
Total number of human hosts	N_H	6 humans	Humans	Bosma et al., 2007	NP
Mortality of reservoir host	μ_R	$5.1\text{x}10^{-3}$ per day	Pigs in agriculture	Peters et al., 2005	NA
Parasite dependent mortality of reservoir host	α_R	0 per day			NA
Average number of worms per infected reservoir host	W_R	36 worms	Fish-borne trematodes in pigs	Lan Anh et al., 2009a	NP
Total number of reservoir hosts	N_R	20 reservoir hosts	Pigs, chickens, dogs, ducks or cat	Bosma, 2007; Tai et al., 2004	NP
Relative reduction proportion of transmission to humans	p_H	1			NP
- Transmission to human	r_H	$1.0\text{x}10^{-8}$ humans per metacercaria per day		(Appendix 2)	NP
Relative reduction proportion of transmission to reservoir host	p_R	1			NP
- Transmission to reservoir host	r_R	$7.2\text{x}10^{-6}$ reservoir hosts per metacercaria per day		(Appendix 2)	NP

Table 1 shows the direction and minimum amount of change per parameter that leads to R<1 based on how parameters appear in the equation for R. A decrease of the relative reduction proportion for transmission to snails and fish (p_S, p_F) from 1 to <0.14 leads to R<1. Parameters that can lead to an R<default, but not to R<1 within biological plausible values are: the number of worms per human and reservoir host (W_H, W_R), the number of humans and reservoir hosts (N_H, N_R) and the relative reduction proportion for transmission to humans and reservoir hosts (p_H, p_R). The other parameters do not appear in the equation for R: changing those parameters does not influence the estimate of the NGM and the R compared to the default situation.

Table 2. Next Generation Matrix (see also Appendix C.1) for the default situation. The expected number of hosts infected per infected host.

To	From			
	Snail	Fish	Human	Reservoir host
Snail	0	0	5914	3.81
Fish	22.0	0	0	0
Human	0	0.0000373	0	0
Reservoir host	0	0.0261	0	0

Control strategies

1. The application of single chemotherapy of humans does not change the R as compared to the default situation (R=1.92). The percentages of infected snails, fish and reservoir hosts decline during the first 15 years after implementation followed by an increase for about 80 years until the percentages are the same as before control. Humans become FZT-free immediately after chemotherapy, but the percentage infected humans increases to the same level as before the implementation in about 80 years (Fig. 2). A single chemotherapeutic treatment leads to similar results if applied to reservoir hosts or both humans and reservoir hosts.

2. The application of continuous chemotherapy of either humans or reservoir hosts leads to a decreased R of respectively 1.30 and 1.69 (Table 3). If humans are treated, the percentage infected humans remains 0, and the percentage infected reservoir hosts decreases from 61% to 38% in the new equilibrium that is reached about 20 years after implementation (Fig. 2 and Table 3). When only reservoir hosts are

treated (Table 3) the percentage infected humans decreases from 50% to 47%. In order to reduce R<1, e.g. 100% of humans and at least 54% of reservoir hosts should be treated continuously (Fig. 3). If both all humans and all reservoir hosts are continuously treated, no transmission takes place (R=0) and thus all host types become uninfected (Table 3).

Table 3. Effect of chemotherapy on the reproduction ratio R and the percentage of infected hosts in stable equilibrium.

Control strategy	Parameter(s) that were set to 0 at t=0	Symbol	R	% Infected hosts at equilibrium			
				I_S	I_F	I_H	I_R
Default situation (no control)			1.92	13	65	50	61
Single chemotherapy							
Humans	Infected humans	$I_{H(0)}$	1.92	13	65	50	61
Reservoir hosts	Infected reservoir hosts	$I_{R(0)}$	1.92	13	65	50	61
Humans and reservoir hosts	Infected humans and reservoir hosts	$I_{H(0)}$, $I_{R(0)}$	1.92	13	65	50	61
Continuous chemotherapy							
Humans	Transmission to humans, infected humans	r_H, $I_{H(0)}$	1.30	2	25	0	38
Reservoir hosts	Transmission to reservoir hosts, infected reservoir hosts	r_R, $I_{R(0)}$	1.69	9	57	47	0
Humans and reservoir hosts	Transmission to humans, reservoir hosts and infected humans and reservoir hosts	r_H, r_R, $I_{H(0)}$, $I_{R(0)}$	0	0	0	0	0

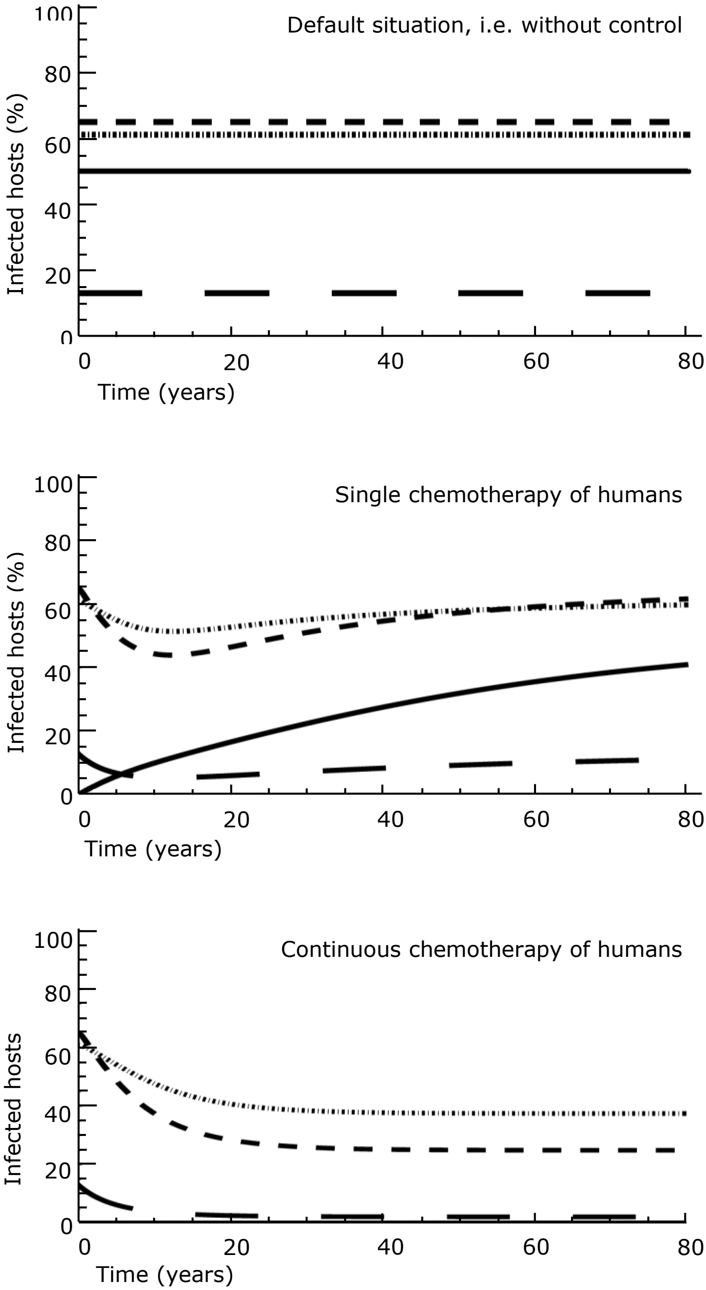

Figure 2. Percentage of infected hosts during a period of 80 years for the default situation without control, and control strategies single and continuous chemotherapy of humans = snail, = fish, = human, and = reservoir host.

3. Control of snails: both decrease in density of snails and increase in mortality of snails (Table 1) do not appear in the equation for R and therefore do not change R. However, density of snails may influence the transmission to snails. A reduction to <14% of the default transmission rate to snails may lead to R<1. An increase in snail mortality may reduce the infectious period of snails and with that the transmission to fish. A reduction to <14% of the default transmission rate to fish leads to R<1.

4. Stocking weight of fish should be >25 g (default is 0.5 g) to reduce R<1. This weight is equivalent to an additional period of >54 days in which fish should be kept FZT-free. To reduce R<1, minimum fish weight at stocking can be lower when combined with continuous chemotherapy of all humans, i.e. 14 g (Fig. 4).

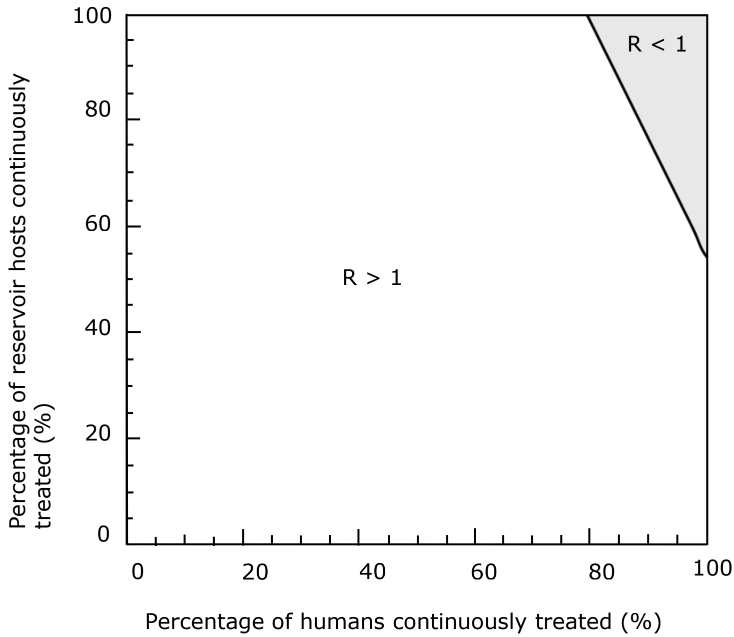

Figure 3. Effect of combining control strategies: continuous chemotherapy of humans and reservoir hosts. Line represents R=1.

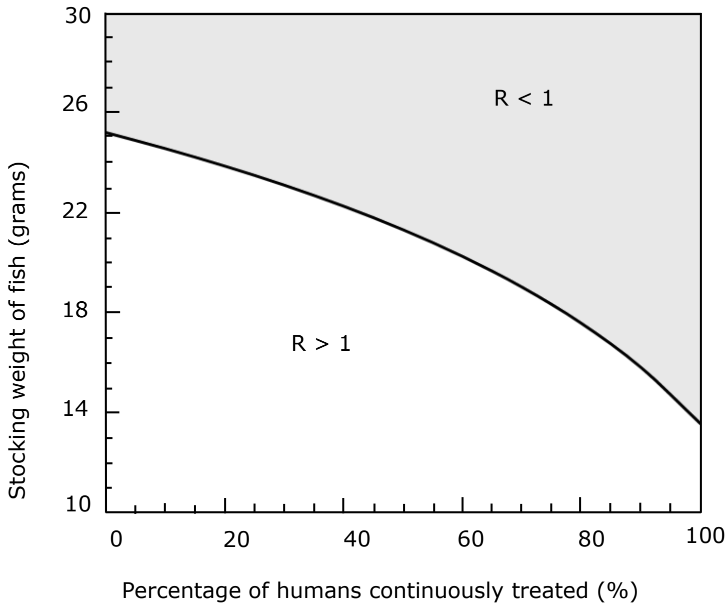

Figure 4. Effect of combining control strategies: continuous chemotherapy of humans and delayed stocking of fish. Line represents R=1.

Discussion

The presented model gives insight into how and to what extent control strategies change the dynamics of FZTs on IAA farms. As expected, the model indicates that FZTs may persist (R>1) in the absence of control strategies. After implementing control strategies either (i) R and percentages infected hosts in the equilibrium do not change and FZTs persist (ii) R becomes smaller, but not below 1, the new equilibrium has lower proportions of infected hosts, and FZTs persist or (iii) R becomes smaller than 1, and all hosts become FZT-free in the new equilibrium.

The effect of chemotherapeutic treatment of definitive hosts depends on the frequency of treatments and on the type(s) of hosts treated. A single treatment does not change the equilibrium percentages of infected hosts because treated hosts become re-infected. Continuous chemotherapeutic treatment of humans leads to FZT-free humans but not to eradication of FZTs, because both humans and other definitive hosts have the capacity to maintain the life-cycle independent of the status of the other. This has also been modelled for the zoonotic blood trematode *Schistosoma japonicum* (Williams et al., 2002), and may explain why chemotherapy of humans did not lead to FZT eradication in areas with a history of FZT treatment in humans. An example is Thailand, where humans are treated for liver flukes since 1960 (WHO, 2011). Eradication of FZTs is possible if (part of) both types of hosts are treated simultaneously. Treatment of reservoir hosts is possible (Anh et al., 2010b) and does not restrict consumption of reservoir hosts (EMEA, 1996). However, wild reservoir hosts (e.g. fish-eating birds) are difficult to treat. The contribution of wild reservoir hosts to the total transmission by reservoir hosts should be quantified to estimate if treating domesticated reservoir hosts in addition to treating humans can be sufficient to eliminate FZTs.

A change in density and/or mortality of snails may change the transmission rates and thus percentages infected hosts in the state of equilibrium. In the field, a decrease in density of snails has led to less infected fish (Clausen et al., 2012a). The relation between density of susceptible individuals and R is often assumed to be linear (De Jong et al., 1994, McCallum et al., 2001). If that holds for snails, FZTs can be eliminated by decreasing density of snails the same as transmission to snails, i.e. below 14% of its default value. An increase in mortality of snails may lead to a decrease in infected snails because it reduces the infectious period of snails (described for schistosomes, Anderson and May, 1979). FZTs may be eliminated if mortality of snails is changed as the reciprocal of the change in transmission to fish, i.e. an increase of 7.14 (1/0.14) times the default value. Field studies are needed to validate these relations. More knowledge about the mechanics of snail control may increase insight into the effect of control measures on FZTs and other zoonotic trematodes with snails as intermediate host, e.g. the intestinal fluke *Fasciolopsis buski* that affects 17 million humans worldwide and has water plants as second intermediate hosts rather than fish (Hopkins, 1992).

A delay in stocking of fish of 54 days more than the traditional 2 to 7 days (Thien et al., 2009), may lead to eradication of FZTs, corresponding to stocking at 25 g instead of 0.5 g. In this scenario, humans may become FZT-free after 20 years or more. If all humans are treated in addition, fish weight at stocking can be 14 g and humans become FZT-free immediately. Treating fish of 25 g which chemotherapeutics to make them FZT-free may also eradicate FZTs. However, the eco-toxicological effects of treating fish are unknown. The most effective drug studied against FZTs in fish, praziquantel (Van et al., 2009), is the only drug available against FZT infections in humans (Soukhathammavong et al., 2011), therefore resistance development in FZTs should be prevented. Delayed stocking and/or treatment of fish may also control other fish-borne zoonotic pathogens in carps or other fish species.

The new equilibria are reached 20 to 80 years after implementation of control, which makes monitoring in the field difficult. The short-term effects that are detected weeks or months after implementation may not predict the eventual equilibrium. Studies that monitor for years may not detect the equilibria either, because circumstances (e.g. the management on a farm) may change.

Assumptions in the model may differ from the situation in the field. The model describes transmission on 1 farm, whereas in the field transmission between farms occurs through e.g. water canals and free roaming reservoir hosts (Dung et al., 2010, Lan Anh et al., 2009a). Adding this transmission to the model requires substantial adaptation of the model. Other different field situations require just a change in default value(s). For example, in an area with mainly liver flukes, parasite dependent mortality of humans may be > 0 because infection in humans is associated with severe worm-load dependent clinical symptoms leading to fatalities (Qian et al., 2011, Wang et al., 2004).

To conclude, several control strategies were identified that could led to eradication of FZTs. It now remains to be evaluated which combination of control measures is attractive to farmers and policy makers in order to test those in the field.

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Appendix A: Ordinary Differential Equations (ODEs)

The equation for the rate of change per day of proportion infected humans is (Eq. A.1):

$$\text{A.1: } \frac{dI_H}{dt} = -\mu_H \, I_H(t) - \alpha_H \, I_H(t) + \frac{r_H \, e^{[-\mu_W \, \tau_W]} \, M \, N_F \, A \, I_F(t)(1 - I_H(t))}{N_H}$$

In which:

$-\mu_H \, I_H(t)$	= natural human mortality
$-\alpha_H \, I_H(t)$	= parasite dependent human mortality
r_H	= transmission to humans (metacercaria becomes worm)
$e^{[-\mu_W \, \tau_W]}$	= proportion of worms surviving the pre-patent period in humans
$\frac{M \, N_F \, A}{N_H}$	= total number of metacercariae per human
$I_F(t)(1 - I_H(t))$	= proportion infected fish multiplied by proportion susceptible humans

The equation for the rate of change per day in proportion infected reservoir hosts is similar to the equation for humans (Eq. A.2):

$$\text{A.2: } \frac{dI_R}{dt} = -\mu_R \, I_R(t) - \alpha_R \, I_R(t) + \frac{r_R \, e^{[-\mu_W \, \tau_W]} \, M \, N_F \, A \, I_F(t)(1 - I_R(t))}{N_R}$$

The equation for the rate of change in proportion infected snails per day has 2 modifications compared to the equations for humans and reservoir hosts (Eq. A.3):

$$\text{A.3: } \frac{dI_S}{dt} = -\mu_S \, I_S(t) - \alpha_S \, I_S(t) + \frac{r_S \, \gamma \, e^{[-\tau_S \, (\mu_S + \alpha_S)]} \, ((W_H \, N_H \, I_H(t)) + (W_R \, N_R \, I_R(t))) \, (1 - I_S(t))}{N_S \, A}$$

The part of the equation that describes a decrease in infected snail hosts is similar. Modifications are:

$((W_H\ N_H\ I_H(t)) + (W_R\ N_R\ I_R(t)))$ = total number of worms from 2 host types instead of 1, i.e. humans and reservoir host

γ = number of eggs shed per worm

The rate of change in proportion infected fish per day is similar to that of humans and reservoir hosts (Eq. A.4):

$$\text{A.4: } \frac{dI_F}{dt} = -\mu_F\ I_F(t) - \alpha_F\ I_F(t) + \frac{r_F\ e^{[-\tau_F(\mu_F+\alpha_F)]}\ C\ N_S\ A\ I_S(t)(1 - I_F(t))}{N_F\ A}$$

Appendix B: Transmission rates

In a stable endemic equilibrium, the rate of change of infected snails, fish, humans and reservoir hosts rate is zero (B.1).

$$\text{B.1: } \left.\begin{aligned} \frac{dI_S}{dt} &= 0 \\ \frac{dI_F}{dt} &= 0 \\ \frac{dI_H}{dt} &= 0 \\ \frac{dI_R}{dt} &= 0 \end{aligned}\right\} for\ I_S = I_S^*,\ I_F = I_F^*,\ I_H = I_H^*,\ I_R = I_R^*$$

The estimated transmission rates (r_S, r_F, r_H, r_R) calculated using the ODEs from Appendix A are (Eq B.2 - B.5):

$$\text{B.2: } r_S = p_S\left(-\frac{N_S\ A\ I_S^*\ (\mu_S + \alpha_S)}{(-1 + I_S^*)\ \gamma\ (W_H\ I_H^*\ N_H + W_R\ I_R^*\ N_R)e^{[\tau_S\ (-\mu_S - \alpha_S)]}}\right)$$

= $1.8 \cdot 10^{-7}$ snails per egg per day

$$\text{B.3: } r_F = p_F\left(-\frac{N_F\ I_F^*\ (\mu_F + \alpha_F)}{(-1 + I_F^*)\ C\ I_S^*\ N_S e^{[\tau_F\ (-\mu_F - \alpha_F)]}}\right)$$

= $8.8 \cdot 10^{-5}$ fish per cercaria per day

$$\text{B.4: } r_H = p_H\left(-\frac{N_H\ I_H^*\ (\mu_H + \alpha_H)}{(-1 + I_H^*)\ M\ I_F^*\ N_F\ A\ e^{[-(\mu_W\ \tau_W)]}}\right)$$

= $1.0 \cdot 10^{-8}$ humans per metacercaria per day

$$B.5: r_R = p_R \left(- \frac{N_R\, I_R^*\, (\mu_R + \alpha_R)}{(-1 + I_R^*)\, M\, I_F^*\, N_F\, A\, e^{[-(\mu_W\, \tau_W)]}} \right)$$

= 7.2·10^{-6} reservoir hosts per metacercaria per day

The *p* represents the relative reduction proportion of transmission in a new situation with control strategies compared to the default situation without control measures. Eq. B.2–B.5 are substituted in Eq. A.1-A.4 and C.1.

Appendix C: Next Generation Matrix (NGM) and *R*

The Next Generation Matrix (NGM) is the transition matrix from 1 generation of infected hosts to the next (C.1).

$$C.1: \mathrm{NGM} = \begin{pmatrix} 0 & 0 & II & III \\ I & 0 & 0 & 0 \\ 0 & IV & 0 & 0 \\ 0 & V & 0 & 0 \end{pmatrix}$$

In which:

$$I = I_F(t + \delta t) = \frac{r_F\, e^{[\tau_F\, (-\mu_F - \alpha_F)]}\, C}{\mu_S + \alpha_S}$$

$$II = I_S(t + \delta t) = \frac{r_S\, \gamma\, e^{[\tau_S\, (-\mu_S - \alpha_S)]}\, W_H}{\mu_H + \alpha_H}$$

$$III = I_S(t + \delta t) = \frac{r_S\, \gamma\, e^{[\tau_S\, (-\mu_S - \alpha_S)]}\, W_R}{\mu_R + \alpha_R}$$

$$IV = I_H(t + \delta t) = \frac{r_H\, e^{[-\mu_W\, \tau_W]}\, M}{\mu_F + \alpha_F}$$

$$V = I_R(t + \delta t) = \frac{r_R\, e^{[-\mu_W\, \tau_W]}\, M}{\mu_F + \alpha_F}$$

Columns represent from left to right infected snails, fish, humans, and reservoir hosts. Rows represent hosts that are infected in the next generation ($I(t+\delta t)$=NGM$xI(t)$). Each interaction between hosts results either in no transmission (0), or transmission (a matrix element). Matrix elements I-V are derived from Eq. A.1-A.4 and are the product of the number of newly infected hosts per day and the length of the infectious period of the host. Matrix element I represents the number of infected fish in the next generation that are a result of interaction with 1 snail during the entire infectious period of the snail. Element II and III represent the number of infected snails, and IV and V represent the number of respectively infected humans and infected reservoir hosts.

$r_F\ e^{[\tau_F\ (-\mu_F-\alpha_F)]}C$ = number of cercariae that become metacercariae: total number of cercariae per snail, multiplied by transmission rate to fish, and proportion of fish surviving the prepatent period

$\frac{1}{\mu_S+\alpha_S}$ = infectious period is 1 divided by mortality of snails

The characteristic equation of NGM is solved:

$$\det\,(NGM - I_{NGM} \cdot \lambda\,) = -1\, \cdot\, II\, \cdot\, IV\, \cdot\, \lambda - I\, \cdot III\, \cdot V\, \cdot\, \lambda +\, \lambda^4 = 0$$

Corresponding eigenvalues are the solutions of the equation:

$$\lambda_1 = 0$$
$$\lambda_2 = I^{\frac{1}{3}} \cdot (II\, \cdot IV + III\, \cdot V)^{\frac{1}{3}}$$
$$\lambda_3 = (-\frac{1}{2} - \frac{1}{2}\sqrt{3}\, \cdot i)\, \cdot\, I^{\frac{1}{3}}\, \cdot\, (II\, \cdot IV + III\, \cdot V)^{\frac{1}{3}}$$
$$\lambda_4 = (-\frac{1}{2} + \frac{1}{2}\sqrt{3}\, \cdot i)\, \cdot\, I^{\frac{1}{3}}\, \cdot\, (II\, \cdot IV + III\, \cdot V)^{\frac{1}{3}}$$

The dominant real eigenvalue of the NGM (the one with the highest real part) is the reproduction ratio R:

$$R = I^{\frac{1}{3}} \cdot (II\, \cdot IV + III\, \cdot V)^{\frac{1}{3}}$$

NGM elements I-V (C.1) can be substituted in this equation to obtain an equation for R.

The eigenvector that corresponds to the dominant eigenvalue represents how, from top to bottom, snails, fish, humans and reservoir hosts are distributed over the typical infected individual. Values are obtained by substituting values of Table 1 and transmission rates of Appendix B, resulting in:

$$\begin{pmatrix} \frac{(II \cdot IV + III \cdot V)^{\frac{2}{3}}}{I^{\frac{1}{3}} \cdot V} \\ I^{\frac{1}{3}} \frac{(II \cdot IV + III \cdot V)^{\frac{1}{3}}}{V} \\ \frac{IV}{V} \\ 1 \end{pmatrix} = \begin{pmatrix} 0.079 \\ 0.908 \\ 0.00002 \\ 0.012 \end{pmatrix}$$

Appendix D: Delayed stocking of fish

The r_F in Table 1 represents the average value of transmission to a fish during its life. Most transmission occurs when fish are small, larger fish (> 50 g) do almost not get infected (Boerlage et al., 2011; 2012). Therefore, if fish remain FZT-free before stocking, r_F can be affected by stocking weight (G_{stock}) and much less so by harvesting weight ($G_{harvest}$). This appendix describes the relation between r_F and G_{stock}, $G_{harvest}$ is kept constant.
First, we define growth of fish. Eq. D.1 shows growth rate (*dG(t)/dt*) for fish in grams per day that is plausible according to Hepher (1988).

$$\mathrm{D.1:}\ \frac{dG(t)}{dt} = 0.119\ G(t)^{0.66}$$

Eq. D.1 is based on a default stocking weight ($G_{stock,\ default}$) of 0.5 g, a harvesting weight ($G_{harvest}$) of 800 g and a grow-out period, i.e. time between stocking and harvesting, of 220 days ($T_{stock,\ default}$ = 0, T_{harvest} = 220) (references see Table 1). The relation between G and T is calculated using Eq. D.2.

$$\mathrm{D.2:}\ G(t) = \int_{t\ =\ T_{stock}}^{T_{harvest}} 0.119\ G(t)^{0.66}\ dt$$

The variables G_{stock} and T_{stock} are changed compared to default stocking.
Second, we relate weight of fish (*G*) to average transmission from snail to fish based on experiments ($r_{F\ experiment}$). Eq. D.3 describes the proportion of fish (*F(G)*) with weight *G* that are infected after being exposed to 250 cercariae based on Boerlage et al. (2012):

$$D.3: F(G) = 1 - (1 + 0.8325\, e^{[1.7621 - 0.1288\, G]})^{-\frac{1}{0.8325}}$$

Eq. D.4 describes $r_{F\ experiment}$ for stocking weight ($r_{F\ experiment}(G_{stock})$). Eq. D.3 is integrated over weight G between stocking weight (G_{stock}) and harvesting weight ($G_{harvest}$), divided by exposure dose (250 cercariae) and grow-out period ($T_{harvest}$ - T_{stock}).

$$D.4: r_{F\ experiment}(G_{stock}) = \int_{G=\,G_{stock}}^{G_{harvest}} F(G)\, dG \cdot \frac{1}{250\ \cdot (T_{harvest} - T_{stock}(G_{stock}))}$$

Third, without control, r_F based on the experiment (Eq. D.4; $r_{F\ experiment}$) can be compared to r_F based on the model (Eq. B.3; $r_{F\ model}$) according to Eq. D.5.

$$D.5: \frac{r_{F\ experiment}}{r_{F\ model}} = \frac{2.7\ \cdot 10^{-4}}{8.8\ \cdot 10^{-5}} = 3.07$$

This ratio of 3.07 is used to convert between model and experiment. E.g., if we calculate with the model described in Appendix A – C that for a certain control measure, R = 1 for a certain value of r_F, then we can convert $r_{F\ model}$ to $r_{F\ experiment}$ and calculate what G_{stock} and T_{stock} should be to lead to R < 1.

8

General discussion

Introduction

Fish-borne Zoonotic Trematodes (FZTs) affect human health worldwide. Humans can get infected when they consume raw or undercooked fish. FZTs persist through a life cycle that involves snails, fish and definitive hosts. Transmission of FZTs can take place from fish to definitive hosts (humans and other vertebrates), from definitive hosts to snails and from snails to fish. Although already 40% of all fish for human consumption originates from aquaculture (FAO, 2012), little is known about transmission mechanisms of FZTs in aquaculture systems. Such knowledge may provide insights into how control measures can affect the dynamics of FZTs in aquaculture.

The objective of this thesis was to obtain more insight in transmission mechanisms of FZTs in aquaculture. A mathematical model was developed and used as a tool to interpret observed epidemiological parameters, to guide collection of reliable quantitative data in experiments and field studies and to lead to further understanding of transmission of FZTs in aquaculture. The model was used to compare and discuss control measures to enable science based recommendations and prioritizations for control measures.

First, a mathematical model was developed. Insights obtained during the development of the model were used to design experiments and field studies. An observational study was done to investigate excretion of FZTs from snails in different areas in an integrated agriculture aquaculture (IAA) pond (Chapter 2). Experiments were performed to investigate the effects of fish size on transmission to fish (Chapter 3 and 4), survival of FZTs in fish (Chapter 5) and the effect of density of FZTs and fish on transmission to fish (Chapter 6). The results from these studies were used to update the mathematical model. This final model was used to compare and discuss control strategies (Chapter 7).

This General Discussion starts with a discussion on transmission mechanisms and control in aquaculture. It continues with a section on practical implications for control on IAA farms, integrated control of FZTs and other pathogens and the methods used in this thesis. This chapter ends with options for future research and a summary of the main conclusions.

Transmission mechanisms

A change in transmission rates to snails and fish can have a large effect on the dynamics of FZTs. The mathematical model indicated that FZTs can be eliminated from IAA farms if transmission to either snails or fish is reduced to less than 14% of its original value. The transmission rates to snails and to fish are sensitive to a number of factors.

Transmission to snails starts when infected definitive hosts excrete FZT eggs in their faeces. Faeces can end up in a pond, e.g. when farmers use animal manure to fertilize a pond (Thien et al., 2009), or because rain flushes faeces of free roaming definitive hosts (e.g. cats) into the pond (Clausen et al., 2012b). Habitat use and defecating habits of definitive hosts may have the largest impact on the prevalence of FZTs in snails (Skirnisson et al., 2004), but may not cause variation in prevalence within a pond (Chapter 2). In the pond, snails become infected after eating the FZT eggs (Komiya, 1966). Within this pathway, transmission rates of FZTs to snails can vary based on density of snails and size of the pond as factor for density of snails (Chapter 7). The mortality rate of reservoir hosts (definitive hosts other than humans, e.g. pigs) may affect transmission to snails because a shorter infectious period of definitive hosts may lead to less transmission (discussed for snails in Chapter 7).

Transmission from snails to fish starts when infected snails excrete free-living FZTs (cercariae). The percentage of infected snails is homogeneously distributed throughout the pond (Chapter 2), but fluctuates with time. For example, the distribution of snails by age class may vary between seasons (Chung et al., 1980) and the percentage of infected snails may be lowest in the season with most juvenile snails that did not acquire infections yet (Ewers, 1964). Other characteristics of the snail population that may affect the transmission rates of FZTs from snails to fish include a change in mortality rate, because of a shorter infectious period (Cook et al., 2008; Smith et al., 2012; Chapter 7), and density of snails (Chapter 7).

The next step of transmission to fish is the search of cercariae for fish, followed by penetration and encystment of cercariae in the muscles of fish (Haas, 2003). Once infected, fish remain infected because of the very low mortality rate of FZTs in fish (Chapter 5). Factors that affect transmission rates to fish include the weight of the fish. Transmission is high when fish are 0.5 gram and reduces until nearly 0 when fish are 50 grams or more (Chapter 3 and 4). The reason for this inverse relationship is possibly the penetration process of FZTs through the skin of fish. During growth of fish, there may be a change in the density of clavate cells in the epidermis of the fish (Rhee, 1974) and/or the thickness or composition of the mucous layer on the fish (Chun, 1964). This relation may exist both within and between species, because the prevalence in smaller fish species is higher than in larger fish species (Komiya, 1966; Rim, 2005; Thien et al., 2007). Another characteristic that affects transmission to fish is the number of cercariae to which fish are exposed (Chapter 3), but not the density of cercariae (Chapter 6). Therefore, high water levels during rainy seasons may not affect transmission to fish if it only reduces the density of cercariae. The higher risk of FZTs that is observed in fish during the rainy season (Thien et al., 2007) may be a result of other factors, such as an increase in snail density due to seasonal effects on demographics of snails (Esch and Fernandez, 1994).

A change in transmission to either humans or other definitive hosts can reduce the percentage of infected snails, fish, humans and other definitive hosts but cannot lead to elimination of FZTs (Chapter 7). This is because both humans and reservoir hosts have the capacity to maintain the life cycle of FZTs independent of the status of the other (Chapter 7). The degree of successful transmission of FZTs from fish to humans depends on the behaviour of humans and their cultural habits of eating raw-fish (Macpherson, 2005; Phan et al., 2011; Grundy-Warr et al., 2012). Humans can also affect transmission to reservoir hosts, if they feed fish to the reservoir hosts (e.g. pigs or cats), and to a lesser extent transmission to free roaming and wild reservoir hosts, e.g. by fencing off the pond.

Effect of control measures on transmission

The insights into transmission mechanisms described above can be used to understand how control measures influence the dynamics of FZTs. The effects of the control measures described in the General Introduction of this thesis are discussed here.

Most control measures against FZTs focus on humans (WHO, 2011). They include chemotherapeutic treatment of humans, education of humans on the risks of eating raw fish and the treatment of raw fish products before consumption. These human-focused control measures may change transmission to humans and may control disease in humans, but may not eliminate FZTs in the long term because FZTs can persist through reservoir hosts (Chapter 7). Additional control measures are needed to eliminate FZTs.

Additional control of FZTs could focus on reducing transmission rates to snails, fish or reservoir hosts. Several control methods could be effective, including changing practices around livestock feeding, snail control and weight of fish at stocking. Feeding caged livestock a fish-free diet reduces transmission from fish to reservoir hosts. This may only lead to FZT elimination in combination with other control measures, because FZTs can persist through humans (Chapter 7). Treatment of manure before it is used as pond fertilizer and replacement of manure with commercial fish food affect transmission to snails and may lead to FZT elimination if the reduction in transmission to snails is sufficient (i.e. less than 14% of its original value). The effect of snail control, e.g. by poly-culture with snail predators, may lead to FZT elimination because it may affect transmission to snails and/or to fish (Chapter 7). The reduction in average transmission to fish is sufficient to eliminate FZTs if the fish are kept FZT-free until they weigh 25 g, because transmission reduces as weight of fish increases (Chapter 3 and 4). Both a single chemotherapeutic treatment of 25 g fish (Van et al., 2009) and a delay in stocking of fish in ponds until fish weigh more than 25 g (Chapter 7) can lead to elimination of FZTs. Overall, control of FZTs by measures that affect transmission to snails or to fish sufficiently (<14% of its original value) can lead to elimination of FZTs. Control against hosts other than humans is an important part of the control strategies to eliminate malaria (Feachem et al., 2010; Smith et al., 2012) and it may have to be an important part of control against FZTs, because eliminating FZTs may not possible without additional control measures focused on snails, fish and/or reservoir hosts.

Practical implications for control on IAA farms

There are opportunities for control measures against FZTs to be implemented in aquaculture. The production cycles of fish include: pre-stocking, stocking, grow-out and harvesting. These different stages are more obvious in an “all-in, all-out” system, in which farmers stock a pond with all fish at the same time and harvest all fish at the same time versus a strategy in which fish are stocked continuously and harvest is partial (Phan et al., 2010b). The “all-in, all-out” type of production will serve as example in this section, however the interventions discussed will be applicable to both unless specified.

Before fish are stocked in a pond, the density of snails can be reduced by drying the pond and removing the mud layer on the bottom that contains snails (Clausen et al., 2012b). This intervention can only be applied in “all-in, all-out” systems between 2 production cycles. The stocking of fish in a pond can also be delayed until fish are larger than the usual 0.5 g to reduce transmission to fish (Chapter 7). However, this requires aquaculture in a trematode-free environment during the first part of the production cycle. Such a change in the farming system may require a fundamental change in traditional farming practices that might be economically difficult to realize for the small-scale village farmer.

During the grow-out period while fish are growing to their harvestable size, farmers can perform poly-culture with the snail-predator black carp (*Mylopharyngodon piceus*) (Ben Ami and Heller, 2001) to reduce the density of snails, which may affect transmission to snails (Chapter 7). A single chemotherapeutic treatment administered to fish after they reach a weight of 25 g may also lead to elimination of FZTs (Chapter 7). The advantage of these two methods, compared to “delayed stocking”, is that FZTs can be eliminated without adaptations in the farming system. A disadvantage to the chemotherapeutic treatment is that the only chemotherapeutic that leads to 100% FZT-free fish, praziquantel, is also the only chemotherapeutic available against FZT infections in humans (Soukhathammavong et al. 2011). Resistance to praziquantel should be prevented because it would possibly jeopardize treatment in humans when this resistance would carry over to the stages of trematodes that occur in humans. The timing of applying praziquantel is not clear if farmers do not apply an “all-in, all-out” strategy resulting in a large variation of weights of fish in the pond. A third option, reducing the duration of the grow-out period, may not affect the dynamics of FZTs, because the mortality rate of FZTs in fish is low (Chapter 5).

Harvesting of fish is the last step in the production cycle of fish. Similar to during the grow-out period, fish can be treated with chemotherapeutics just before harvesting. This may lead to trace amounts of praziquantel in the fish at consumption, which are not considered a restriction for human consumption (EMEA, 1996). The advantage of treating fish just before harvesting is that fish will be FZT-free at consumption, whereas there may be some successful transmission of FZTs to fish when fish are treated during grow-out (e.g. at > 25 g). If fish > 50 g are exposed to FZTs, the chances on becoming infected are about 0 (Chapter 3 and 4) and new infections are no longer a concern. This intervention may not be feasible if farmers harvest partial.

Integrated control against several pathogens

Control measures against FZTs may also control other parasites. Co-endemicity (more parasites occurring in one area) and poly-parasitism (more parasites occurring in one host) are common (Prichard et al., 2012). Biological and epidemiological similarities between parasites may provide opportunities to integrate control against several pathogens, which could improve human health with efficient use of resources (e.g. WHO, 2011; Prichard et al., 2012).

Chemotherapeutic treatment of humans is an important control measure against many helminthic infections. Treatment of humans with praziquantel can cure e.g. infections with *Diphyllobothrium* (Muller, 2002) and blood flukes of the genus *Schistosoma* that affect almost 240 million humans worldwide (Doenhoff et al., 2008; WHO, 2013). Control strategies that combine praziquantel and other anthelmintics can cure infections with over 90% of the zoonotic helminths (Hotez et al., 2006; Utzinger and de Savigny, 2006). However, the previously discussed limitations of the use of human chemotherapeutic control may also apply to other helminths: helminths that depend on a life cycle and both have humans and reservoir hosts as definitive hosts may not be eliminated by chemotherapeutic treatment of humans only. To find additional control measures, the life cycles of helminths that co-exist in one area can be studied to identify transmission mechanisms that could be targeted with similar control strategies, such as improved sanitation. Improved sanitation can reduce contamination of ponds with faeces and manure, and therefore reduce transmission of many pathogens. Examples are FZTs and other food-borne zoonotic trematodes that have snails as a first intermediate host, trematodes that have other aquatic species as first intermediate hosts, e.g. the trematode *Diphyllobothrium* that has copepods as first intermediate hosts, and zoonotic bacteria (Petersen and Dalsgaard, 2003).

Use of a mathematical model and transmission experiments

The combination of field studies, experiments and a theoretical model provided insight into the effect of control measures on the dynamics of FZTs in hosts. Furthermore, the potential control measure "delayed stocking" that was developed and tested in this thesis, is an example of how a potential control measure can be obtained by integrating a theoretical study with experiments and observations.

Both mathematical models and (transmission) experiments simplify reality. The advantage of an experiment, compared to an observational study, is that the history of exposure is known so transmission can be quantified accurately (Velthuis et al., 2007). Furthermore, the effect of an investigated factor cannot remain unnoticed in the variation caused by other factors. A disadvantage of a transmission experiment is that extrapolation of results to a field situation may not be straightforward (Velthuis et al., 2007). The advantage of using theoretical models to understand transmission is that the process of developing the model provides insight into the dynamics (De Jong et al., 1995). Furthermore, multiple control measures under different circumstances can be tested relatively easy and in a short time, and results can be extrapolated to other areas and other circumstances because the values of default parameters (i.e. those without control) can be changed. Possibly, the model developed in this thesis can be used for other trematode species with a similar life cycle. Because the results of models are based on a simplification of reality, the model should be validated in the field.

Validation of a model is important because it tests the insights and predictions obtained from the model to see whether they are reliable. For FZTs, new equilibrium percentages of infected snails, fish and definitive hosts established in 20 – 80 years after control measures are applied (Chapter 7). Validation of the model during this period is not easy due to changes and loss to follow-up (see discussion Chapter 7). Furthermore, prevalence data obtained during monitoring may be subject to seasonal variations (Wright, 1971; Wykoff et al., 1965). Possibly the best method to detect an effect of control is by exposing

FZT-free, small fish for a set period in a pond. Transmission rates to these fish are high compared to transmission rates to other hosts and the number of FZTs per fish relates to the exposure dose (Chapter 3). To obtain insight into the eventual equilibrium percentage of infected snails, fish and definitive hosts, monitoring may have to be applied for at least 20 years (Chapter 7).

Future research

This thesis has provided insight into transmission mechanisms of FZTs and discussed possible effects of control measures. Further research can include the following subjects:

- An intervention study can test the effect of delayed stocking in the field and could be used to validate the model.
- Transmission experiments between cercariae and different species of fish can be used to test which fish species are more susceptible for FZTs than others. The penetration of cercariae in fish can be studied to discover the cause of the difference in transmission rate between small and larger fish. Results may possibly help farmers select fish species used in their farms.
- The effect of density and mortality of snails on the transmission rate of FZTs to snails and fish has not yet been quantified. The relationship between these parameters and transmission rates can be used to better understand the effect of snail control on FZT persistence and possibly the effect of snail control on the persistence of other parasites that have snails as intermediate hosts.
- A single treatment of fish with praziquantel may lead to eradication of FZTs. The eco-toxicological effects of the application of praziquantel and the possible development of resistance of FZTs against praziquantel should be investigated. The model (Chapter 7) provides a starting point of comparison of different control strategies against FZTs based on the basic reproduction ratio. A cost-benefit analysis, in which the costs and benefits of the different control strategies are compared, may provide insight into the feasibility of control measures.

Conclusions

The research in this thesis helped to provide more insight into the transmission of FZTs and the infection dynamics of fish-borne trematodes. IAA farms in Vietnam were used as example, but the insights may be extrapolated to other situations in other countries. Still, it might take many years before control can lead to elimination of fish-borne zoonotic trematodes. The following main conclusions can be drawn from this thesis:

- Smaller common carp (0.5 g) get more often and more heavily infected with FZTs compared to larger carp (> 50 g), which rarely acquire new infections.
- FZTs persist in common carp for at least 27 weeks.
- Delayed stocking of fish may lead to FZT elimination.
- Chemotherapeutic treatment of humans as only control measure cannot lead to FZT elimination. FZTs can persist in the absence of infected humans, because both humans and reservoir hosts other than humans have the capacity to maintain the life cycle independent of the status of the other.

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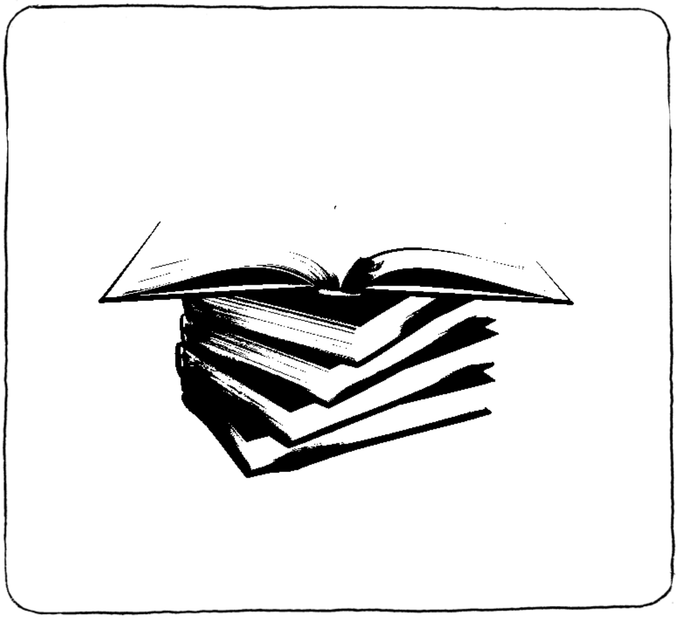

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Summary

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Introduction and objective

Fish-borne zoonotic trematodes (FZTs) are commonly reported. They affect the health of millions of humans worldwide and affect economics in terms of loss of productivity, absenteeism, and health care costs. Furthermore, food safety issues can affect trade. More than 70 species are known to be zoonotic. The life cycle of FZTs involves aquatic snails, fish, and vertebrate definitive hosts like humans, pigs or chickens. Definitive hosts can get infected when they consume raw or undercooked fish. Farmers can manage fish in aquaculture, which may provide opportunities for control of FZTs in fish. Development of such measures requires a better understanding of transmission mechanisms to fish. Furthermore, different control measures target different parts of the life cycle of FZTs and may also be used in combination. A better understanding of how control measures affect transmission dynamics is needed in order to predict which (combination of) control measures may be effective and which may not.

This thesis combines experiments, statistical analysis and mathematical modelling of FZT in aquaculture to improve understanding of the impact of several control measures. The objective is to obtain more insight in transmission mechanisms of FZTs in aquaculture by developing a mathematical model that is used as a tool to interpret observed epidemiological parameters, guide collection of reliable quantitative data in experiments and field studies and might lead to further understanding of transmission of FZTs in aquaculture. The model is used to compare and discuss control measures with the ultimate goal to enable science based recommendations and prioritizations for control measures. All studies in this thesis use transmission of Heterophyidae cercariae between *Melanoides tuberculata* snails and common carp (*Cyprinus carpio*) on Integrated Agriculture-Aquaculture (IAA) farms in Nam Dinh province, Vietnam, as an example to get insight into transmission of FZTs in aquaculture.

Transmission between snails and fish

At first, a mathematical model was build. The process of building the model provided insights that helped designing experiments and field studies to obtain more insight into transmission from snails to fish.

Transmission of FZTs between snails and fish in aquaculture can be split into excretion of FZTs (cercariae) by snails and transmission of cercariae to fish. Chapter 2 describes an observational study on excretion of cercariae by snails. Manure is used as pond fertilizer in IAA farming, thereby enhancing transmission of FZTs from definitive hosts to snails. IAA ponds are typically surrounded by agriculture, a livestock sty for pigs or poultry with outlets to the fish pond, and an access point to the pond for humans. Areas within a pond could vary in trematode egg-load due to the immediate bordering land, and with that cause patchiness in FZT infection in snail populations within a pond. *M. tuberculata* snails were sampled in ponds on IAA farms in Nam Dinh province. There were only very small differences between areas surrounding a pond on risk of snails to be infected with fish-borne trematodes within different pond areas. Control measures targeting specific areas within a pond are therefore not considered potential intervention measures.

Chapter 3 – 6 describe experiments on transmission of FZTs to fish and subsequent survival in fish. One of the factors not always consistent in observational studies is the relation between fish weight and infection probability and/or number of FZTs established. This relation may only become clear from experimental exposure studies, because observational studies lack quantitative information about previous exposure of fish to FZTs. Two transmission experiments have been conducted to quantify the effect of fish weight on the percentage of fish infected and the proportion of FZTs established in fish (Chapter 3 and 4). Both experiments demonstrate an inverse relation between weight of common carp and gain of FZTs. Common carp acquire most FZTs when they are small (i.e. 0.5 g), fish of more than 50 grams hardly acquire new infections. It was concluded that reducing transmission to fingerlings may reduce the amount of FZTs in the environment to a large extent.

Common carp are one of the most preferred freshwater fish species by consumers in Asia, the region where fish-borne zoonotic trematodes (FZTs) like Heterophyidae are most prevalent. The percentage infected fish and number of FZTs per fish at harvesting depends on previous gain and subsequent survival of FZTs. Gain is investigated in Chapter 3 and 4 and survival is investigated in an experiment in Chapter 5. Results showed that the number of trematodes that established in carps did not change significantly until the study ended 27 weeks post exposure. This may imply that Heterophyidae persist in common carps and harvestable carps may still contain about maximum levels of FZTs and are a risk to human health.

Transmission of FZTs to fish in aquaculture takes place in a more-or-less closed designated area where the dimensions of the water depth and surface area of the base of the fish enclosure can be regulated, as well as the number of fish. A different number of fish per designated area may have an effect on the number of trematodes per fish, which may be relevant to human health. Chapter 6 investigates the effect of the density of cercariae and the density of fish on transmission with respect to the volume of water and surface area of the bottom. Neither the prevalence of infection with fish-borne trematodes, nor the number of metacercariae per fish nor the proportion of trematodes established in fish were significantly associated with differences in the density of cercariae or the density of fish. It was concluded that transmission to fish was not affected by changes in the density of either cercariae or fish.

Control measures

The mathematical model developed at first, was updated with results of the experiments and used to get insight into the effect that the following control measures and combinations of those measures may have on dynamics of FZT infection in hosts: chemotherapeutic treatment of humans, definitive hosts other than humans or both, snail control, and delayed stocking of fish (Chapter 7). Single chemotherapy of humans, other definitive hosts or both did not affect the persistence of FZTs. Continuous chemotherapeutic treatment resulted in a lower percentage infected hosts but FZTs persisted. Combined treatment of humans and other definitive hosts, however, could lead to FZT elimination. Snail control may lead to elimination of FZTs. Stocking fish at more than 25 g as compared to 0.5 g that is usual in aquaculture practice, or at more than 14 g in combination with treating all humans, led to elimination of FZTs. The new equilibria, FZT-free or not, were often established 20 to 80 years after implementing the control strategy.

General conclusions

The research in this thesis provided insight into the transmission mechanisms of FZTs and the effects of several control measures on the infection dynamics of FZTs. This research may be helpful in designing appropriate control strategies against FZTs in order to improve human health. IAA farms in Vietnam were used as example, but the insights may be extrapolated to other situations in other countries as well. Still, it might take many years before control can lead to elimination of fish-borne trematodes.

The following main conclusions can be drawn:

- Smaller common carp get more often and more heavily infected with FZTs compared to larger carp (> 50 g), which rarely acquire new infections.
- FZTs persist in common carp for at least 27 weeks.
- Delayed stocking of fish may lead to FZT elimination.
- Chemotherapeutic treatment of humans as only control measure cannot lead to FZT elimination. FZTs can persist in the absence of infected humans, because both humans and reservoir hosts other than humans have the capacity to maintain the life cycle independent of the status of the other.

It remains to be evaluated which combination of control measures is attractive to farmers and policy makers.

Samenvatting

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Introductie en doelstelling

Zoönotische trematodes in vissen (FZTs) zijn parasieten die wereldwijd de gezondheid van miljoenen mensen beïnvloeden. Dit heeft financiële gevolgen door verminderde productiviteit van geïnfecteerde mensen, en leidt tot meer ziekteverzuim en hogere ziektekosten. Tevens kunnen FZTs in vis een nadelig effect hebben op handel i.v.m. verminderde voedselveiligheid. Er zijn meer dan 70 soorten FZTs bekend. In de levenscyclus van FZTs zijn drie types gastheren betrokken: aquatische slakken, vissen en eindgastheren zoals de mens, varkens, kippen en andere vertebraten. Eindgastheren raken besmet door het eten van rauwe of onvoldoende gekookte vis (voornamelijk zoetwatervis). De aquacultuur biedt mogelijkheden om FZT-infecties te voorkomen, omdat viskwekers door middel van managementmaatregelen het productieproces kunnen beïnvloeden. Voor het ontwikkelen van zulke controlemaatregelen is echter meer inzicht nodig in de mechanismes van transmissie. Verschillende controlemaatregelen of combinaties daarvan grijpen in op verschillende delen van de levenscyclus van FZTs. Een beter begrip van hoe controlemaatregelen de dynamica van transmissie tussen gastheren beïnvloedt, is nodig om te bepalen welke (combinatie van) controlemaatregelen effectief zijn en welke niet.

Dit proefschrift combineert experimenten, statistische analyses en een wiskundig model van FZTs in de aquacultuur om meer inzicht te krijgen in de impact van controlemaatregelen. Eerst is een wiskundig model ontwikkeld om effecten van epidemiologische parameters te interpreteren en de opzet van dataverzameling in experimenten en veldstudies te optimaliseren om zo transmissie van FZTs in de aquacultuur te onderzoeken. Ter verbetering van het model zijn de resultaten van deze studies erin verwerkt. Vervolgens is het model gebruikt om controlemaatregelen te vergelijken en te bediscussiëren. Het ultieme doel is om, gebaseerd op wetenschappelijk onderzoek, aanbevelingen te doen over controlemaatregelen. Als voorbeeld is gebruik gemaakt van transmissie van FZTs uit het geslacht Heterophidae, vanuit de slak *Melanoides tuberculata* naar de karper *Cyprinus carpio*, in vijvers van bedrijven in de provincie Nam Dinh in Vietnam waarop landbouw en aquacultuur gecombineerd wordt, zogenaamde IAA bedrijven.

Transmissie tussen slakken en vissen

Allereerst is een wiskundig model gemaakt. Het proces van het modelleren heeft geleid tot inzichten die gebruikt zijn om experimenten en veldstudies te ontwerpen voor het verzamelen van informatie over de FZT-transmissie van slak naar vis. Deze transmissie kan worden opgesplitst in de excretie van FZTs (cercariae) door slakken en de transmissie van cercariae naar vissen. Hoofdstuk 2 beschrijft een observationele studie over de excretie van cercariae door slakken. Op IAA bedrijven worden de uitwerpselen van dieren en mensen vaak gebruikt om de visvijver te bemesten. Op deze manier is visvoer niet nodig. Echter, uitwerpselen kunnen eieren van FZTs bevatten, waarmee transmissie van FZTs van eindgastheer naar slak wordt bevorderd. Visvijvers op IAA bedrijven worden meestal omringd door verschillende zaken of activiteiten, zoals bijvoorbeeld akkerbouw, een stal voor varkens of pluimvee met een afvoerbuis naar de visvijver en een plaats waar mensen toegang hebben tot de vijver. Door deze verschillen in gebruik van land rondom een vijver zou er variatie kunnen zijn in aantal eieren van FZTs op verschillende plaatsen in de visvijver en dus ook tussen FZT-infecties in de slakpopulatie. FZT-infecties in *M. tuberculata* slakken zijn op verschillende plaatsen in vijvers van IAA bedrijven in de provincie Nam Dinh in Vietnam gemeten. Er waren slechts kleine verschillen tussen het type land rondom vijvers en het risico dat slakken besmet waren met FZTs. Het uitvoeren van controlemaatregelen tegen FZTs in slakken in een specifiek gedeelte van een vijver wordt daarom als niet effectief beschouwd.

In hoofdstuk 3 tot en met 6 zijn experimenten over transmissie van cercariae naar vis en de overlevingsduur van FZTs in de vis (metacercariae) beschreven. De relatie tussen het gewicht van vis en de kans dat een vis besmet is c.q. het aantal FZTs per vis is niet altijd consistent beschreven in de publicaties van observationele studies. Zulke studies geven geen informatie over de blootstelling van de vis aan FZTs voorafgaande aan een observatie en de enige manier om deze relatie helder te krijgen, is het doen van experimentele studies. Twee transmissie-experimenten zijn uitgevoerd om de relatie tussen het gewicht van een vis en de besmettingskans en de proportie van FZTs die zich in vissen hebben gemanifesteerd te meten (hoofdstuk 3 en 4). Uit beide experimenten is duidelijk geworden dat de relatie tussen het gewicht van karpers en het besmet raken met FZTs omgekeerd evenredig is. Karpers worden besmet met FZTs als ze klein zijn (0.5 g), terwijl vissen van meer dan 50 gram nauwelijks geïnfecteerd raken. Het verminderen van de transmissie van FZTs naar kleine vissen zou de totale hoeveelheid FZTs behoorlijk kunnen reduceren.

De karper is een van de meest favoriete zoetwatervissen voor Aziatische consumenten. Tevens is Azië de regio waar FZTs (zoals Heterophyidae) het meeste voorkomen. Het percentage geïnfecteerde vis en het aantal FZTs per vis in geoogste vis hangt niet alleen af van de, in hoofdstuk 3 en 4 beschreven, toename in FZTs tijdens het leven van de vis, maar ook hoe lang FZTs vervolgens in de vis overleven (hoofdstuk 5). Het aantal FZTs per vis verminderde niet significant gedurende 27 weken na besmetting. Dit betekent dat Heterophyidae lang in karpers kunnen overleven. Karpers bevatten daarom tijdens het oogsten mogelijk nog steeds FZTs en vormen zo een risico voor de humane gezondheid.

In de aquacultuur vindt transmissie van FZTs naar vissen plaats in een min of meer gesloten ruimte, bijvoorbeeld een visvijver, waarin waterdiepte, wateroppervlakte en het aantal vissen gereguleerd kunnen worden. Een verschil in dichtheid van vissen zou een effect kunnen hebben op het aantal trematodes per vis. Het effect van dichtheid van zowel cercariae als vissen op de transmissie is beschreven in hoofdstuk 6. Dichtheid is gedefinieerd als aantal cercariae en aantal vissen per watervolume en bodemoppervlakte. Zowel het percentage FZT-geïnfecteerde vissen, het aantal FZTs per vis als de proportie van de FZTs dat zich succesvol manifesteert in de vis, zijn niet significant afhankelijk van dichtheid van cercariae of vissen. Er is geconcludeerd dat transmissie naar vissen niet wordt beïnvloed door veranderingen in dichtheid van cercariae of vissen.

Controlemaatregelen

In hoofdstuk 7 is het wiskundige model beschreven, geüpdatet met de resultaten van de experimenten en de veldstudie. Het model is gebruikt om inzicht te krijgen in het effect dat de volgende controlemaatregelen en combinaties van maatregelen kunnen hebben op de dynamica van FZT-infecties in de gastheren: medicatie van mensen of eindgastheren anders dan mensen of allebei, maatregelen tegen slakken en het uitzetten van zwaardere vissen in de vijver. Het eenmalig toedienen van een anthelminthicum heeft geen gevolgen voor de overleving van FZTs. Het continu toedienen leidt tot een lager percentage geïnfecteerde gastheren, maar FZTs blijven wel aanwezig. Als echter zowel voldoende mensen als voldoende niet-humane eindgastheren tegelijkertijd continu behandeld worden, kunnen FZTs niet persisteren; bijvoorbeeld met een doorlopende behandeling van alle mensen en 54% van de niet-humane eindgastheren. Controlemaatregelen tegen slakken kunnen ook leiden tot het uitsterven van FZTs; dit kan door bijvoorbeeld dichtheid van slakken te verminderen of de sterfte van slakken te bevorderen. Gewoonlijk worden vissen op een gewicht van 0.5 g in de vijver gedaan, en daarmee mogelijk blootgesteld aan FZTs. Als dit wordt uitgesteld tot een gewicht van 25 g, kunnen FZTs uitsterven. Dit is ook mogelijk als vissen in de vijver worden uitgezet op een gewicht van 14 g en alle mensen doorlopend behandeld worden met een anthelminthicum. Pas 20 tot 80 jaar nadat een controlemaatregel is ingesteld, wordt een evenwicht bereikt in de percentages geïnfecteerde gastheren. Dit geldt zowel voor controlemaatregelen die leiden tot een lager percentage geïnfecteerde gastheren als controlemaatregelen die leiden tot het uitsterven van FZTs.

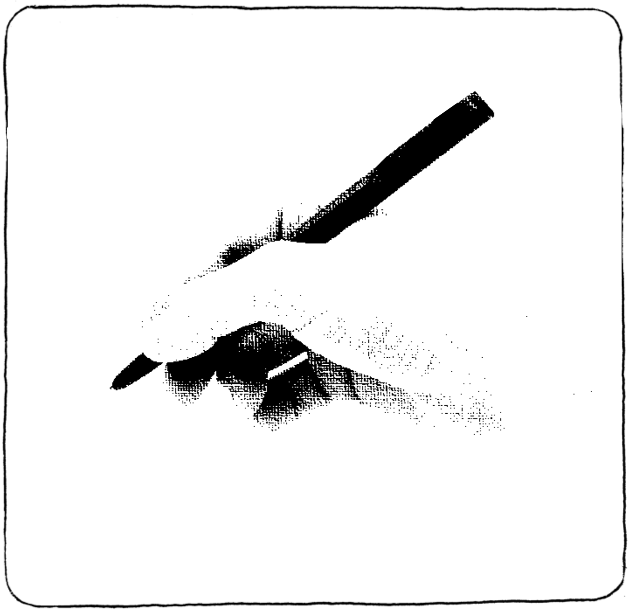
Algemene conclusies

Dit proefschrift heeft meer inzicht gegeven in de transmissie van FZTs in de aquacultuur en de effecten van verschillende controlemaatregelen op de dynamica van infectie met FZTs. Resultaten van het onderzoek beschreven in dit proefschrift kunnen gebruikt worden om effectieve controlemaatregelen tegen FZTs te ontwikkelen om daarmee humane infecties te voorkomen. IAA bedrijven in Vietnam zijn gebruikt als voorbeeld, maar de inzichten kunnen ook geëxtrapoleerd worden naar situaties in andere landen. Het zou echter nog vele jaren kunnen duren voordat controlemaatregelen leiden tot eliminatie van FZTs.
De belangrijkste conclusies van dit proefschrift zijn:

- Kleinere karpers worden vaker geïnfecteerd en met meer FZTs dan grotere karpers (>50 g), die nauwelijks besmet worden.
- FZTs kunnen minstens 27 weken overleven in vissen.
- Vissen later, en dus op hoger gewicht, in de vijver uitzetten, kan leiden tot het uitsterven van FZTs.
- Het behandelen van mensen met anthelminthica als enige controlemaatregel kan nooit leiden tot het uitsterven van FZTs. FZTs kunnen voortbestaan in de afwezigheid van (besmette) mensen, omdat zowel mensen als niet-humane eindgastheren de levenscyclus van FZTs onafhankelijk van elkaar in stand kunnen houden.

Het is nu aan viskwekers en beleidsmakers om te bepalen welke (combinatie van) controlemaatregelen zij willen gebruiken.

About the author



Curriculum vitae

Annette Simone Boerlage was born on August 13th 1983 in Stedum, the Netherlands. She graduated in 2001 from the Nienoord College in Leek and started her BSc in Animal Sciences at Wageningen University, the Netherlands in that year. She continued with an MSc in Aquaculture and Fisheries at Wageningen University, which she completed in 2007. During her MSc, she completed a thesis on the welfare of African catfish at the Aquaculture and Fisheries Group, Wageningen University; a research internship studying the bacteriology of Pangasius catfish at the department of Aquatic Biology and Pathology at Can Tho University, Vietnam; and a major thesis on eel virology at the Laboratory for Fish, Shellfish and Crustacean Diseases of the Central Veterinary Institute of Wageningen UR. During her MSc she was involved in teaching BSc and MSc courses as a student assistant for the Experimental Zoology Group of Wageningen University. In 2007, Annette began her PhD thesis at the Quantitative Veterinary Epidemiology Group of Wageningen University, focusing on the epidemiology of fish-borne zoonotic parasites. Currently, Annette holds a post-doc position on aquatic epidemiology in the Canada Excellence Research Chair Program at the Atlantic Veterinary College, University of Prince Edward Island, Canada.

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Awards

Second prize oral at 7th symposium on Diseases in Asian Aquaculture. 7th symposium on Diseases in Asian Aquaculture (DAAVII), 22-26 July 2008, Taipei, Taiwan.

First prize poster at 8th symposium on Diseases In Asian Aquaculture. 8th symposium on Diseases in Asian Aquaculture (DAAVIII), 21-25 November 2011, Mangalore, India.

First prize oral at 16th WIAS Science Day. 16th symposium of Wageningen Institute of Animal Sciences (WIAS), 2 February 2012, Wageningen, Netherlands.

WIAS training and supervision plan

The basic package (3.0 ECTS)

WIAS introduction course, WIAS, Wageningen	2008
Course on philosophy of science and ethics, WGS, Wageningen	2007

Conferences, seminars and workshops (5.1 ECTS)

The 7th symposium on diseases in Asian aquaculture, Taipei, Taiwan	2008
International symposium on catfish aquaculture in Asia, Can Tho, Vietnam	2008
WIAS science day, Wageningen, The Netherlands	2008
WIAS science day, Wageningen, The Netherlands	2009
WIAS science day, Wageningen, The Netherlands	2010
The 8th symposium on diseases in Asian Aquaculture, Mangalore, India	2011
WIAS science day, Wageningen, The Netherlands	2012

Presentations (8.0 ECTS)

Oral presentation at the 7th symposium diseases in Asian aquaculture, Taipei, Taiwan	2008
Poster at the international symposium on catfish aquaculture in Asia, Can Tho, Vietnam	2008
Poster at the WIAS science day, Wageningen, The Netherlands	2008
Poster at the 8th symposium on diseases in Asian aquaculture, Mangalore, India (3x)	2011
Oral presentation at the 8th symposium on diseases in Asian aquaculture, Mangalore, India	2011
Oral presentation at the WIAS science day	2011

In-depth studies (15.0 ECTS)

Multivariate mathematics applied, Wageningen University	2007
Modern statistics for the life sciences, Wageningen University	2008
Introduction to infectious disease modelling and its applications, London School of hygiene and tropical medicine, UK	2011

Professional skills support courses (4.0 ECTS)

PhD competence assessment, WSG, Wageningen University	2008
Course techniques for scientific writing, WGS, Wageningen University	2010
Writing the PhD thesis and its propositions, WIAS, Wageningen University	2011
Mobilizing your –scientific- network, WGS, Wageningen University	2011
Communication with the media and the general public, WGS, Wageningen University	2011

Didactic skills training (1.2 ECTS)

Supervising groups for the BSc course ‘introduction animal sciences’, Wageningen University	2011

Education and training total: 36 ECTS

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