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# The Effect of Saprotrophic Fungi on the Development and Hatching of *Fasciola hepatica* Eggs

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The aim of this study was to determine the effect of 6 common soil fungi species: *Alternaria alternata* (Fr.) Keissl., *Aspergillus candidus* Link, *Penicillium chrysogenum* Thom, *P. commune* Thom, *Trichothecium roseum* (Pers.) Link and *Ulocladium* sp., on the hatching of miracidia, i.e., free living larvae of liver fluke (*Fasciola hepatica)*. To this end, the eggs of *F. hepatica* were incubated in water in the presence of one of the aforementioned fungi species and in tap water (control) at a temperature of 26°C. At the 15$^{th}$ day of incubation we determined the number of nonembryonated, embryonated and hatched eggs. We observed different degrees of antagonistic influences by the tested fungal strains on the development of *F. hepatica* eggs. Among the examined fungi, the strongest ovistatic effects were exhibited by *Trichothecium roseum*, *Penicillium chrysogenum* (R-3) and *P. commune*. The study showed no morphological damage to the shells of the *F. hepatica* eggs which may suggest a biochemical basis of antagonistic interactions by the fungi associated with the activity of fungal enzymes, mycotoxins and antibiotics. Low or no activity of peptide hydrolases in *Penicillium chrysogenum* and *P. commune* in the API ZYM test suggests their insignificant role in the degradation of shell proteins of *F. hepatica* eggs.

Key words: *Fasciola hepatica*, embryogenesis, saprotrophic fungi, eggs, enzymatic activity.

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The liver fluke (*Fasciola hepatica*), the etiological factor of fasciolosis, is a cosmopolitan digenean trematode parasitizing the liver and bile ducts of domesticated and wild herbivorous mammals as well as humans. On a global scale, fasciolosis is a veterinary problem which results in considerable economic loss, mainly in the breeding of cattle and sheep, due to a lower production of milk, wool and meat (SPITHILL & DALTON 1998). In 2003 the prevalence rate of fasciolosis in Polish slaughter cattle was 12.1% (LIS 2005), while related loss, due to elimination of livers, was estimated at over 1 million PLN in 1997 (DERYŁO & SZILMAN 1999). Since then the prevalence of fasciolosis in Polish cattle has greatly decreased, but in north-eastern Poland it is still significant and remains at a constant level (MICHALSKI & ROMANIUK 2000).

About 180 million people worldwide are at risk of infection with *F. hepatica*, and according to various estimates from 2.4 million (WHO – WORLD HEALTH ORGANIZATION 1995) to 17 million people (HOPKINS 1992) may be infected. The high risk of infection occurs mainly in people inhabiting endemic areas. Fasciolosis is a serious health problem in South America (Bolivia, Peru, Chile and Ecuador) and in Egypt, Iran and Vietnam. In Europe, the majority of cases of fasciolosis have been observed in France, Portugal and Spain (MAS-COMA *et al*. 2005).

Infection of the definitive host occurs via the gastrointestinal tract after consumption of the infective stage of the parasite – metacercariae. Juvenile flukes in the small intestine pass through the peritoneal cavity to the liver parenchyma and then

to the bile ducts, where they reach sexual maturity. One fluke lays up to 25 thousand eggs a day which are then expelled with the host's feces. Embryogenesis takes place in an aquatic environment and is completed with the hatching of the first larval stage – the miracidium. This form is an infective stage for the intermediate host – amphibious snails from the Lymnaeidae family, being the site of development for subsequent larval stages – sporocysts, rediae and cercariae. The latter leave the body of the intermediate host and encyst on aquatic plants as metacercariae.

Embryogenesis and the hatching of larvae are significantly influenced by temperature, light and oxygen tension. The rate of embryogenesis is influenced by various physical and chemical factors. It has been shown that X-rays lengthen the duration of embryogenesis and result in a lower level of hatched miracidia, while laser rays shorten embryogenesis and accelerate hatching of larvae (BIELECKI 1986). Likewise, exposure of *F. hepatica* eggs to an extremely low frequency magnetic field (ELFMF) results in acceleration of hatching of the liver fluke larvae (KOŁODZIEJCZYK *et al*. 2010). It has been demonstrated that pesticides extend the duration of embryogenesis and cause a decrease in *F. hepatica* hatchability (BIELECKI 1985; CHRISTIAN *et al*. 1985).

Abiotic factors in the ecosystem determine the occurrence of mutually interacting organisms. This dynamic system includes a number of interspecies interactions which determine the number and vitality of species. Populations of organisms, including helminth parasites of humans and animals, may be reduced via various forms of antagonisms, for example those involving micro-fungi (LÝSEK 1978; MIZGAJSKA 1994). To date researchers have focused on the ovicidal effect of saprotrophic fungi on the development of eggs, e.g. *Ascaris suum* and *A. lumbricoides*. Particularly significant results have been observed for the following fungal strains: *Penicillium frequentans*, *Stachybotrys chartarum*, *Fusarium oxysporum*, *F. culmorum*, *Trichocladium asperum*, *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) *P. lilacinus*, *Metarhizium flavoviride*, *M. anisopliae*, *Trichothecium roseum*, *Aspergillus versicolor*, *A. niger*, *Metacordyceps chlamydosporia* (=*Verticillium chlamydosporium*), *Mucor hiemalis* and *Pochonia chlamydosporia* (LÝSEK & KRAJČI 1987; LÝSEK & ŠTĚRBA 1991; BASUALDO *et al*. 2000; KOŁODZIEJCZYK *et al*. 2001; KU NA-GRYGIEL *et al*. 2001a, 2001b; CIARMELA *et al*. 2002; ARAÚJO *et al*. 2008). The ovicidal effects of *Pochonia chlamydosporia* have also been observed in the eggs of other species; *Trichuris vulpis* (SILVA *et al*. 2010), *Toxocara vitulorum* (BRAGA *et al*. 2010) and *Taenia saginata* (ARAÚJO *et al*. 2009). Hyphal penetration of *Pochonia chlamydosporia* has also been observed in the eggs of *Schistosoma mansoni* (BRAGA *et al*. 2008a) and *Fasciola hepatica* (BRAGA *et al*. 2008b; DIAS *et al*. 2012). It is also possible that during *F. hepatica* embryogenesis the eggs are infected by fungus-like organisms (FLOs) (KIZIEWICZ 2006).

Data on effects of other microfungi on the development of flukes outside the host are only fragmentary. Hence in this research, our aim was to examine the effect of common saprotrophic soil fungi on the development of eggs of *Fasciola hepatica*.

## Material and Methods

Adult forms of *F. hepatica* were collected from bile ducts in naturally infected cattle. The study material were eggs isolated from the uterus of flukes, and the following strains of soil fungi: *Ulocladium* sp. (R-20), *Penicillium chrysogenum* (R-73), *Penicillium chrysogenum* (R-3), *Trichothecium roseum* (9B-15), *Alternaria alternata* (R-53), *Penicillium commune* (R-5) and *Aspergillus candidus* (A-29). The cultivation of fungi was carried on PDA medium at 23-25°C for 21 days.

In order to determine the enzymatic activity of the fungi, we selected strains of the *Penicillium* genus most common in the environment according to our own studies: *P. chrysogenum* (R-3; R-73) and *P. commune* (R-5). We used the API-ZYM® test (bioMerieux, Lyon, France) to semi-quantitatively determine the activity of 19 hydrolytic enzymes; alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phoshohydrolases, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosoaminidase, α-mannosidase and α-fucosidase. Hydrolytic activity was determined in nanomoles of hydrolyzed substrate, in a color scale from 0 to 5 provided by the manufacturer indicating the reactions; 0 – negative reaction, 1 – 5 nM, 2 – 10 nM, 3 – 20 nM, 4 – 30 nM and 5 – 40 nM and more.

*F. hepatica* eggs (0.8 ml) were incubated in tap water (5 ml) in Petri dishes (with an area of 8 $cm^2$ each) in the presence of individual fungal species. *F. hepatica* eggs incubated in tap water without the presence of fungi were used as the control. Incubation was carried out at 26°C for 15 days. On the 10th day of incubation, the light cycle of the cultures of eggs was darkened to avoid premature larvae hatching. On the 15th incubation day the eggs of *F. hepatica* experimental and control cultures were exposed to light for 1.5 h. Then *F. hepatica*

egg suspensions were collected from the control and experimental cultures (0.1-0.4 ml) and were studied microscopically. Observations of 100 randomly collected eggs were carried out 3 times to determine the stage of development. The eggs were classified as nonembryonated – in the zygote stage (A), embryonated – during embryonic development (B), and (C) eggs after hatching – with opened operculum.

Statistical evaluation of differences in the influence of the tested strains of fungi on *F. hepatica* larvae hatching were performed using Chi-square tests and STATISTICA 9 software. Differences were deemed significant at $P<0.05$.

## Results and Discussion

In this study we found varying degrees of antagonist effects of the tested fungal strains on the development of *F. hepatica* eggs, expressed in the different ratios of nonembryonated eggs, embryonated eggs and eggs after hatching (with opened operculum) (Fig. 1). Among the tested fungal species, the most inhibitory effects on embryonic development and hatching of larvae of *F. hepatica* were exerted by three species; *Trichothecium roseum*, *Penicillium chrysogenum* (R-3) and *P. commune* (R-5), as manifested in an approximately 3-times higher percentage of non-embryonated eggs in comparison with the control and a total lack of developed larvae (miracidia). These differences were statistically significant. A somewhat weaker inhibitory effect on the development of eggs and larvae hatching was exerted by the strain *Penicillium chrysogenum* (R-73). The presence of *Alternaria alternata* and *Ulocladium* sp. resulted in a delay of embryonic development of *F. hepatica,* as manifested by a higher proportion of embryonated eggs – from cleavage to miracidium stage (33% and 49%). The least inhibitory effect on the hatching of *F. hepatica* larvae was shown by *Aspergillus candidus*, as evidenced by the number of hatched miracidia, 3-times lower compared to the control (Fig. 1C). The number of nonembryonated *F. hepatica* eggs incubated in the presence of *A. candidus* was similar to the control (Fig. 1A), and approximately 3 times more eggs were observed during embryonic development – the majority in the morula stage (Fig. 1B).

We found varying degrees of antagonist influence of the tested fungal strains on the development of *F. hepatica* eggs. Among the studied species of fungi, *Trichothecium roseum, Penicillium chrysogenum* (R-3) and *P. commune* had the significantly strongest ovostatic influence. We found no morphological damage to egg shells of *F. hepatica*, which may suggest a biochemical basis of the antagonistic effects of the tested fungal strains. The effectiveness of the observed antagonistic activity of the examined fungi may have resulted from a number of direct or indirect factors (LÝSEK 1978). The latter are related to the metabolic activity of fungi. Antagonism based on biochemical activity of fungi plays an important role in the bioregulation of populations of many hel-

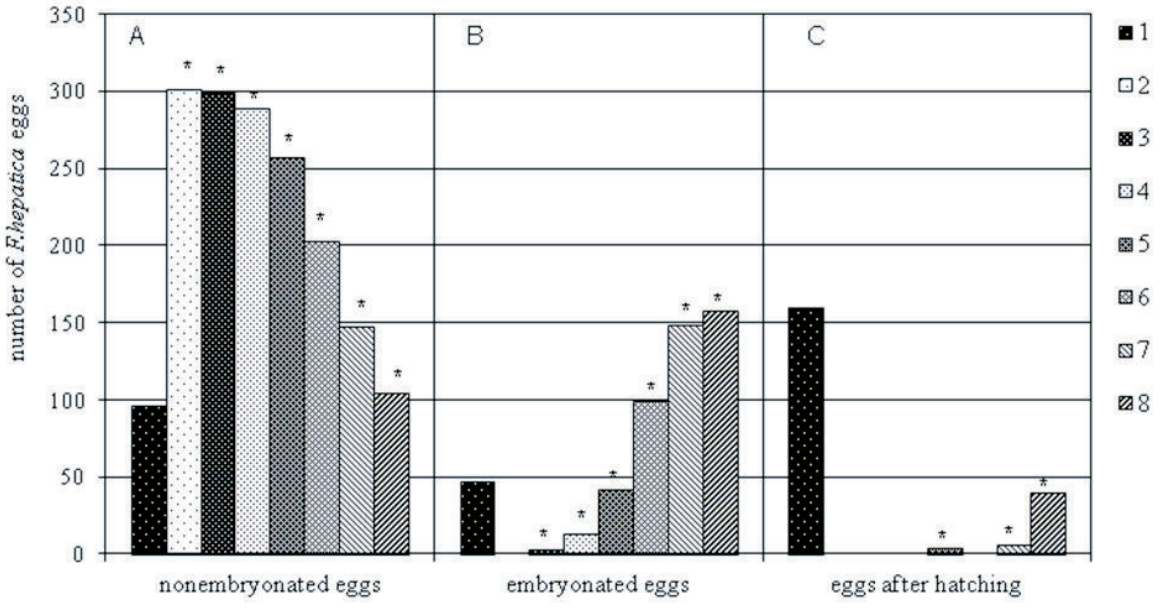


Fig. 1. The number of *Fasciola hepatica* eggs at the 15th day of incubation in control (1) and in the presence of fungi: 2 – *Trichothecium roseum*; 3 – *Penicillium chrysogenum* (R-3); 4 – *Penicillium commune*; 5 – *Penicillium chrysogenum* (R-73); 6 – *Alternaria alternata*; 7 – *Ulocladium* sp.; 8 – *Aspergillus candidus*.
A – nonembryonated eggs; B – embryonated eggs; C – eggs after hatching; * – differences statistically significant at $P<0.05$.

minths that are responsible for parasitoses in humans and animals (LÝSEK 1978; BASUALDO *et al*. 2000; KOŁODZIEJCZYK *et al*. 2001; KU NA-GRYGIEL *et al*. 2001a, 2001b; CIARMELA *et al*. 2002).

Specific biochemical properties of microorganisms were examined by a standard API ZYM test. An examination of 19 hydrolytic enzymes has shown that the tested strains of *P. chrysogenum* and *P. commune* were capable of biosynthesizing alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and the naphthol-AS-BI-phoshohydrolases, α-galactosidase, β-glucosidase and N-acetyl-β-glucosaminidase. In addition, strains of *P. chrysogenum* showed activity of α-mannosidase (R3), leucine arylamidase and β-galactosidase (R3 and R73). The highest activity in all strains of *Penicillium* was found for β-glucosidase and N-acetyl-β-glucosaminidase (Table 1).

It is known that the egg shell of *F. hepatica* is formed by a sclerotin or quinone-tanned protein (FAIRWEATHER *et al*. 1999). In this study, among the tested peptide hydrolases (leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin and chymotrypsin), only leucine arylamidase in *P. chrysogenum* showed some (but weak) activity. This observation does not exclude the participation of other proteolytic enzymes in the degradation of *F. hepatica* egg shells, as the three fungal species (*Trichothecium roseum*, *Penicillium chrysogenum* and *P. commune*) that most inhibited the development of eggs have been reported to have proteolytic activity (DOMSCH & GAMS 1993; RODRÍGUEZ *et al*. 1998; ÖZKAN & ERTAN 2012; PAPAGIANNI 2013).

The important role of mycoenzymes (of *Penicillium frequentans*, *P. verrucosum* var. *cyclopium, Rhizoctonia solani*) – lipases and proteases – in the destruction of eggs has been demonstrated in a phytopathogenic nematode *Globodera rostochiensis* (MAZURKIEWICZ-ZAPAŁOWICZ 2002) the egg shells of which are composed of protein, chitin and lipid layers. In this study, the lipolytic activity of all strains of *Penicillium* (Table 1) may have caused changes in the permeability of cell membranes in the developing embryos and interfered with the course of embryogenesis of *F. hepatica* eggs. Lipolytic activity has also been observed for *Trichothecium roseum* (SIPAHIOGLU & HEPERKAN 2000; JANDA *et al*. 2009).

In addition to mycoenzymes, an inhibitory effect on the development of larvae of *F. hepatica* may be exerted by secondary fungal metabolites, primarily mycotoxins. In this study, significant inhibition of cleavage, resulting in the complete absence of larvae of *F. hepatica*, was shown in the presence of *T. roseum* and *P. commune*, whose

Table 1

Production of 19 hydrolases by fungal species *Penicillium chrysogenum* and *Penicillium commune* in the APIZYM® test (bioMerieux). Rounded up means in the test color scale

| Hydrolases | | *Penicillium* strains | | |
|---|---|---|---|---|
| | | *P. chrysogenum* (R 73) | *P. chrysogenum* (R 3) | *P. commune* (R 5) |
| 1. | Alkaline phosphatase | 2.5 | 3 | 1 |
| 2. | Esterase (C4) | 0.5 | 1 | 1 |
| 3. | Esterase lipase (C8) | 0.5 | 1 | 0,5 |
| 4. | Lipase (C14) | 0 | 0 | 0 |
| 5. | Leucine arylamidase | 0.5 | 1 | 0 |
| 6. | Valine arylamidase | 0 | 0 | 0 |
| 7. | Cystine arylamidase | 0 | 0 | 0 |
| 8. | Trypsin | 0 | 0 | 0 |
| 9. | Chymotrypsin | 0 | 0 | 0 |
| 10. | Acid phosphatase | 2 | 2 | 1 |
| 11. | Naphthol-AS-BI-phosphohydrolase | 2 | 2 | 1 |
| 12. | α-galactosidase | 1 | 2 | 1,5 |
| 13. | β-galactosidase | 3 | 3 | 0 |
| 14. | β-glucuronidase | 0 | 0 | 0 |
| 15. | α-glucosidase | 0 | 0 | 0 |
| 16. | β-glucosidase | 4 | 5 | 3 |
| 17. | N-acetyl-β-glucosaminidase | 4 | 5 | 3 |
| 18. | α-mannosidase | 0 | 1 | 0 |
| 19. | α-fucosidase | 0 | 0 | 0 |

many strains are toxigenic (ANTIPOVA *et al*. 2011; KOTESWARA *et al*. 2011; MCCORMICK *et al*. 2011). *Trichothecium roseum* produces trichothecenes which have an irritating effect on the skin and mucous membranes of animals. The ovostatic effect of *P. commune* on *F. hepatica* eggs is probably also related to the activity of other mycotoxins. Most of the strains of this species produce ochratoxin A-OTA (ANTIPOVA *et al*. 2011; PIOTROWSKA 2012) the toxicity of which is manifested by the inhibition of the activity of numerous enzymes and in disturbances in carbohydrate and protein metabolism (BAUDRIMONT *et al*. 1997). OTA also causes changes in the activity of oxidative enzymes in the developing eggs of *Ascaris suum* (KU NA-GRYGIEL *et al*. 2001b). Also in our study, incubation of *F. hepatica* eggs in the presence of mycelia of *P. commune* caused significant inhibition of embryogenesis. This observation indirectly indicates the inhibitory effect of metabolites of *P. commune* on the development of *F. hepatica* eggs.

Apart from mycotoxins, an ovostatic effect is also exhibited by antibiotics – other secondary metabolites produced by fungi. In this study, antibiosis may have been manifested in the inhibition of embryogenesis of *F. hepatica* in the presence of *P. chrysogenum* strains, commonly known producers of penicillin. The β-lactam antibiotics block the activity of transpeptidases, leading to disturbances in the permeability of cell membranes.

Our study indicates an important role of the tested fungi in modifying the dynamics of embryonic development of *F. hepatica* and at the same time indicates how little is known and how insufficiently used are the abilities of microorganisms to modify dynamic inter-species networks. The possibility of using these microorganisms in the bioregulation of populations of different helminth species requires further interdisciplinary research.

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