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Recovery of *Fascioloides magna* (Digenea) population in spite of treatment programme? Screening of *Galba truncatula* (Gastropoda, Lymnaeidae) from Lower Austria

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ABSTRACT

During the past decade, *Fascioloides magna*, the large American liver fluke, has spread within free-living deer in wetlands of the Danube in Lower Austria. The aim of this study was to determine the current infection rates with *F. magna* and other digenean parasites in the intermediate host snail *Galba truncatula* from risk areas in Lower Austria. A total of 3444 *G. truncatula* were collected and examined microscopically for the presence of digenean trematodes. A set of randomly selected snails and isolated trematode stages were also investigated molecular biologically by PCR and sequencing. Digenean parasites were detected with a prevalence of 2.41% (1.83% Paramphistomoidea; 0.46% Echinostomatoidea; 0.09% Strigeida; 0.06% Plagiorchiida). *F. magna* was found with an overall prevalence of 0.23%, which may indicate a recovery of the parasite population in spite of an ongoing triclabendazole treatment programme. Moreover, high risk areas and a seasonality of infections were observed.

1. Introduction

The large American liver fluke *Fascioloides magna* (Digenea: Fasciolidae) is an important parasite of wild and domestic ruminants in North America, causing considerable economic losses. The life cycle of *F. magna* involves free-living parasitic stages as well as endoparasitic stages and two successive hosts (Pybus, 2001). *F. magna* has repeatedly been introduced to Europe, with first records from the late 19th century from Italy. In Europe, definitive hosts are red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) (Erhardová-Kotrlá and Kotrly, 1968) and the snail species *Galba truncatula* (Erhardová-Kotrlá, 1971; Erhardová-Kotrlá, 1968) and *Radix peregra* (Faltýnková et al., 2006) are confirmed natural intermediate hosts. Dependent on temperature and snail species, the development inside the snail host takes about 40–58 days (Swales, 1935; Erhardová, 1961) and includes sack-like sporocysts, two redial generations, and a cercarial stage with a long, unforked tail.

Austria is a classic example where the parasite has been probably repeatedly introduced over many years, but become endemic only relatively recently and only in a small area. In Austria, the parasite was first detected in fallow deer in a game reserve close to the Danube in Lower Austria (Pfeiffer, 1983). The first infected free-living cervids were found in autumn 2000 in the Danube floodplain forests of Fischamend, Lower Austria (Winkelmayer and Prosl, 2001). During the past 10 years, the parasite spread through wild red and roe deer populations in riparian zones east

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of Vienna, particularly in Fischamend, Regelsbrunn, Orth and Mannswörth (Winkelmayer and Prosl, 2001; Ursprung, 2002; Ursprung and Prosl, 2011). Cervidae endemic in Austria can be severely affected by the parasite, as its migrations within the tissue lead to severe liver damage and may cause death of the hosts. In contrast, livers of red deer show chronic changes and, dependent on the parasite load, the animals become weakened or die spontaneously without marked symptoms (Erhardová-Kotrlá and Kotrly, 1968). Already one year after the first finding in the wild a monitoring and control programme was launched in the wetlands south of the Danube. In brief, free-living deer was treated with Fasinex® (Novartis Animal Health Inc.), a 10% suspension of triclabendazole, admixed with the winter feed, thus administering 10–15 mg triclabendazole per kg body weight within a period of six days. Treatment was performed 3–4 times (2001–2005), respectively 2 times (since 2006) per annum. Consequently, the number of infections in definitive hosts markedly decreased within the following years (Ursprung et al., 2006). Also, a very low prevalence of *F. magna* in *G. truncatula* was recorded, when a first screening was conducted in 2004/2005 (Hörweg et al., 2011). However, a complete eradication of *F. magna* in Austria was not achieved. Starting in 2006, a shift to the northern side of the Danube and a significant resurgence of *F. magna* infections in definitive hosts was recorded, with prevalences up to 72% (Ursprung and Prosl, 2011).

Thus, in 2008 the current study was initiated, in order to analyse the infection dynamics in the intermediate host – after the observed relapse in wild ruminants. In a GIS-based risk analysis of the Danubian wetlands east of Vienna the region around Orth was identified as a risk area (Reckendorfer and Groiss, 2006). Therefore we determined the infection rates in *G. truncatula* with *F. magna* and other digeneans in this risk area north of the Danube. Furthermore, we performed an evaluation of the distribution of infected snails and of a possible seasonality of infections, as well as investigations on population dynamics of the intermediate host snail *G. truncatula*. The screening was performed by microscopy as well as by molecular methods.

2. Materials and methods

2.1. Study sites

The sampling was carried out from April to October 2008 on three distinct locations in the area Orth/Danube (Lower Austria) in the Danube wetlands east of Vienna, Austria. As shown in Fig. 1, the first sampling site, Entenhaufen, was located at the estuary area of the river Große Binn. This location is characterized by plane river banks, small vegetation and an abundance of periodically flooded, sandy-muddy sampling islets. At location 2 (Märchenteich), bank borders flanking an "animal crossing" (a small bridge), were sampled. Traverses like this divide the river Kleine Binn into several basins (Fig. 1). Dependent on the average water level, only a very small (approx. 1 m^2) muddy area was available for sampling. However, whenever the water level was low enough, additionally extensive, sandy-muddy bank borders had been exposed. The river section of the Kleine Binn sampled at location 3 (Neubruchwiese) is located between the "animal crossing" and the junction of the river Rohrhaufenarm (Fig. 1). This river section exhibited the highest flow velocity of all sampling sites (Reckendorfer, 2000). Very dense vegetation flanking both sides of the precipitous river bed made it difficult to access the small, stony sampling sites. Approximately every 14 days, *G. truncatula* were manually collected and stored at 8 °C or in 75% EtOH at room temperature until further examination.

2.2. Parasitological examinations

A total of 3444 *G. truncatula* were collected, measured, dissected and examined under a stereomicroscope for the presence of digenean trematodes. Parasitic stages were photographed by light microscopy and stored at −20 °C. Every tenth microscopically parasite-free snail was chosen as a random sample and conserved at −20 °C for further molecular biological investigation. Altogether, 61 random samples and 24 microscopically positive samples were also investigated by PCR and sequencing. The latter included 9 representatives of the superfamily Echinostomatoidea, 7 of the superfamily Paramphistomoidea, 4 mixed infections and 4 undefined helminths.

2.3. Primer design

Two primer pairs were developed to enable both, the detection of trematodes in general and the specific detection of *F. magna* in *G. truncatula* (Table 1). For primer design, rDNA sequences of various control species (e.g. trematodes, nematodes, snails) were aligned to determine sections with suitable properties. Then common criteria of primer design (e.g. Burpo, 2001), such as similar melting temperatures for forward- and reverse primers, reasonable GC-contents and primer lengths, no self-annealing and/or to other primer, etc. were applied and the most promising primer pairs were chosen. The expected sizes of the amplicons were approx. 430 bp (TremF/TremR), 580 bp (MagnaF/MagnaR) and 124 bp (FASC_f/FASC_r), respectively. All primers were purchased from Eurofins MWG Operon (Germany).

To examine the specificities of the primers, all primer pairs were tested twice with DNA from different reference species. The primer pair TremF/TremR amplified controls of *F. magna*, *F. hepatica* and *Paramphistomum* sp., controls of *Taenia saginata*, *Diphyllobothrium latum* and *G. truncatula* were not amplified. MagnaF/MagnaR amplified *F. magna*- and *F. hepatica*-DNA, controls with *Paramphistomum* sp., *T. saginata* and *G. truncatula* remained negative. For final differentiation between the two closely related species *F. magna* and *F. hepatica*, the *F. hepatica* specific primer pair FASC_f/FASC_r (Kaplan et al., 1995; Caron et al., 2011) was included. Test runs with FASC_f/FASC_r and *F. hepatica*-DNA yielded around 124 bp and 248 bp long amplicons. Controls with *F. magna*, *Paramphistomum* sp., *Aspidogaster limacoides*, *Proteocephalus longicollis*, *Diplostomum* sp. and *G. truncatula* remained negative.

Furthermore, to analyze the sensitivity and robustness of the newly developed primer pairs, different amounts of *G. truncatula* DNA were mixed with a total of 1.4 ng *F. magna*-DNA (ratio 1:1707; 1:3414; 1:6060) and tested with