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„Molecular species determination of larval
Digenea as basis for reliable epidemiological
analyses, enhanced biodiversity data and
significant medical risk assessments“

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1. Introduction

1.1. Trematodes

Within the phylum Platyhelminthes, the so-called flatworms, are among others the class of the Cestoda and the class of the Trematoda Rudolphi, 1808, the latter consisting of the two subclasses Aspidogastrea Faust & Tang, 1936 and Digenea Van Beneden, 1858. The subclass of the Digenea contains the orders Paramphistomatiformes Szidat, 1936, Echinostomatiformes (Echinostomida) La Rue, 1957, Strigeiformes (Strigeatida) La Rue, 1957, Opisthorchiformes (Opisthorchiida) La Rue, 1957, and Plagiorchiformes (Plagiorchiida) La Rue, 1957 (Meyers *et al.*, 2000). With their ~18,000 nominal species the Digenea are considered the largest and most specious group of internal metazoan parasites (Cribb *et al.*, 2001). All digenetic trematodes are parasitic. Many of them, e.g. *Fasciola* spp., *Schistosoma* spp., *Paragonimus* spp., *Clonorchis* spp. and *Trichobilharzia* spp., have medical or economical relevance (Prugnolle *et al.*, 2005). They are characterized by complex life cycles with an obligatory change of host and an alternation of sexual and asexual reproduction during their lifespan (Prugnolle *et al.*, 2005). All trematodes use molluscs as intermediate hosts, where the asexual phase takes place and genetically identical larvae are produced. The sexual phase occurs in the vertebrate definitive host (Prugnolle *et al.*, 2005). “Some flukes use paratenic hosts in which immature parasites undergo no morphologic development or reproduction. Flukes may also use amphiparatenic hosts, which are lactating female mammals that would otherwise serve as definitive hosts, but function instead as paratenic hosts by infecting their offspring with immature parasites via breast milk” (Meyers *et al.*, 2000). In order to get into their host, the trematodes have special organs of adhesion or anchorage; generally these are suckers or hooks (Gibson *et al.*, 2002). They use the suckers or hooks to penetrate the host’s skin, enter the body and parasitize the interior, e.g. the intestinal tract, lungs, liver and other internal organs. Generally, parasites with these characteristics are called endoparasites. In contrast, ectoparasites attach themselves to the host’s surface (Cheng 1986; Gibson *et al.*, 2002). Digenean Trematoda belongs consistently to the endoparasites (Cheng 1986). Most digenean flukes are hermaphroditic and produce operculated eggs. The schistosomes are unique among the Digenea in that the sexes are separate (dioecious). The females produce non-

operculate eggs (Cheng, 1986). The majority of the Digenea provide a flat surface and vary in their size up to a length of several inches; among the largest are *Fasciolopsis buski*, which measures up to 7.5 cm, and *Fascioloides magna*, which measures up to 10 cm (Meyers *et al.*, 2000).

Until now, there are only a few studies, which deal with the occurrence and distribution of these families in Europe, especially in Austria (*Trichobilharzia* spp.: (Dvorak *et al.*, 1999; Konecny *et al.*, 1999; Auer and Aspöck 2002; Sattmann *et al.* 2004; Kolářová *et al.*, 2013) ; *F. magna*: (Hörweg *et al.*, 2011; Ursprung and Prosl, 2011; Kasny *et al.*, 2012).

1.1.1. Fasciolidae

The family Fasciolidae belongs to the order Echinostomatiformes (Echinostomida) La Rue, 1957. The family includes several representatives of veterinary or medical relevance, especially *Fascioloides* (*Fascioloides magna* Bassi, 1875), *Fasciola* (e.g. *Fasciola hepatica* Linnaeus, 1758) and *Fasciolopsis* (*Fasciolopsis buski* Looss, 1899).

1.1.1.1. Classification and morphology

Beginning in the late 18th century, the classification of Fasciolidae was originally based on morphological characteristics, such as sucker arrangements (Dujardin, 1845 [cit. in Cribb *et al.*, 2001]), cercarial morphology (Cable, 1947 [cit. in Gibson, 1987]) or life cycle characteristics (van Beneden, 1858 [cit. in Grove, 1990]). Members of the family Fasciolidae Railliet, 1895 have a flattened and elongated body, mostly with an armed tegumentum. The anterior is formed as a head pivot and the mouth opening can have a thorny crown. The oral and ventral suckers are located closely to one another. The cercariae are simple-tailed and have a gymnocephalic shape (Pybus, 2001). The adults of *F. magna* have an oval dorsoventrally flattened body with two suckers, oral and ventral. They are reddish-brown coloured and covered by a tegumentum (Erhardová-Kotrlá, 1971). *F. hepatica* is also dorsoventrally flattened and has an oral and a ventral sucker (Eckert *et al.*, 2008). *F. buski* is a large, leaf-shaped, dorsoventrally flattened fluke with two suckers, whereby the ventral sucker is larger than the oral sucker (Roberts *et al.*, 2009). Nowadays phylogenetical studies combine the knowledge on the morphology with molecular data. Faust (1929) defined the superfamily Echinostomatoidea to which belongs the family Fasciolidae, based on flame-cell

patterns and life-history data (Kostadinova and Jones, 2005). La Rue expanded the approach of Faust by establishing a classification based on the larval characteristics (1957 [cit. in Kostadinova and Jones, 2005]). La Rue's superfamily Echinostomatoidea contained nine families, including the Fasciolidae (Kostadinova and Jones, 2005). To clarify the phylogenetic relationships of the echinostomatoid families, a few more studies were performed. By combining molecular, morphological and life-history data, Cribb *et al.* (2001) underpin the concept of Echinostomatoidea with four families, the Echinostomatidae, Philophthalmidae, Fasciolidae and Cyclocoelidae. The family Fasciolidae includes six genera, most of them are medically and economically relevant, including *Fasciola* Linnaeus, 1758 (*F. hepatica*), *Fascioloides* Ward, 1917 (*F. magna*), *Fasciolopsis* Looss, 1899 (*F. buski*), *Parafasciolopsis* Eijsmont, 1932, *Protofasciola* Odhner, 1926a and *Tenuifasciola* Yamaguti, 1971. The Fasciolidae comprise the majority of genera of liver flukes of domestic animals (Jones, 2005 a).

1.1.1.2. Life cycle

Digenean trematodes have a complex life cycle and an obligate alternation of sexual and asexual reproduction. The asexual phase takes place in the first intermediate host, the sexual phase in the final host. The life cycles are characterized by a spectrum of potential intermediate and final hosts, as well as by the number of hosts. In the following, the life cycle of *F. hepatica* will be described. The life cycle of *F. hepatica* was elucidated in the 1880s by Leuckart and Thomas (Naquira-Vildoso & Marcial-Rojas, 1971). Adult flukes measure up to 30 x 13 mm. They have an average life span in humans of up to 10 years (Andrews, 1999; Chen and Mott, 1990). The mature worms produce numerous immature eggs, which are operculated. "Egg production is generally inversely proportional to worm burden" (Meyers *et al.*, 2000). They measure around 140 x 75 µm and are yellowish-brown (Andrews 1999; Ash and Orihel, 2007). After migrating from the bile ducts into the small intestine, the eggs are excreted with the feces of their final host (see Fig. 1[1]). The external surface of the eggshell is very robust, but smooth. In watery surroundings, after a maturation phase of 10–15 days in suitable climatic conditions (15°C to 25°C) (Lapage, 1968 [cit. in Meyers *et al.*, 2000]), the miracidiae hatch from the eggs (see Fig. 1[2]). In unsuitable conditions the eggs do not hatch, but remain viable for several months (Lapage, 1968 [Meyers *et al.*, 2000]). Light stimulates the eggs of *F. hepatica* to release the miracidiae. Therefore, the

miracidiae of *F. hepatica* have light stimulated apical glands, secreting proteolytic enzymes that digest parts of the eggshell (Buzzell, 1983 [cit. in Fried & Graczyk, 2000]). The developed miracidiae are about 130 µm in length (Andrews, 1999) and are finally released through the different osmotic pressure levels between in- and outside. The miracidiae have an oblong, oval shape and are transparent. The epithelial cells are covered with long cilia. Miracidiae possess glandular and excretory structures, a large cephalic ganglion and a cone-shaped apical papilla with sensory receptors and gland openings. They also have numerous sensory organs, and typically possess eyespots as well. The miracidiae subsequently infect snails of the genus *Lymnaea* (means Lymnaeidae including *Galba*), which are often found in marshes and swamps (Andrews, 1999; Ash and Oriel, 2007). After finding the proper intermediate host (see Fig. 1[4]) the miracidiae usually penetrate the snail's head-foot region or its mantle. The miracidia are able to anchor in the host tissue with the help of a gland secretory system. Within the snails the miracidiae undergo several developmental stages (see Fig. 1[4a, 4b, 4c]). Within 6-7 weeks they transform into sporocysts. In the liquid-filled inner of the mother-sporocysts are a determined number of germ cells, which develop into daughter sporocysts, redia or directly into cercariae, which are then secreted from the snails (Ash and Oriel, 2007). The cercariae emerge from the host snails and attach to aquatic vegetation, such as watercress and water caltrop nuts. They encyst to form metacercariae, which are infective for mammals within 24 hours (Boray, 1964; Chen and Mott, 1990; Dawes & Hughes, 1964; Naquira- Vildoso and Marcial-Rojas, 1971). In the process of the metacercaria-metamorphosis the parasites loose cercarial structures for example mucoid or penetration glands. The metacercariae of *F. hepatica* show a strong adhesion to the vegetation. They can persist over a time period of about one year, even if they are without nutrition, but the infectivity and vitality decreases over time (Soulsby, 1965). The infection is acquired by ingestion of the metacercariae on the vegetation, but can also occur by ingestion of the metacercariae floating in the water (see Fig. 1[6]) (Ash and Oriel, 2007). The gastro-intestinal system of the definitive host digests the cyst wall of the metacercariae and juvenile worms are released (see Fig. 1[7]). The larval *F. hepatica* migrate through the intestinal wall into the abdominal cavity, enter the liver, and burrow through the parenchyma to enter the bile ducts where the adult worms reach maturity in 3-4 months and begin to secrete eggs. Because of the extra intestinal migration of the larval stages of *Fasciola*, it is not uncommon for these

worms to end up in ectopic locations, including the abdominal wall, lungs, brain and orbit (Ash and Oriel, 2007). *F. hepatica* can survive up to 13 years in humans (Meyers *et al.*, 2000).

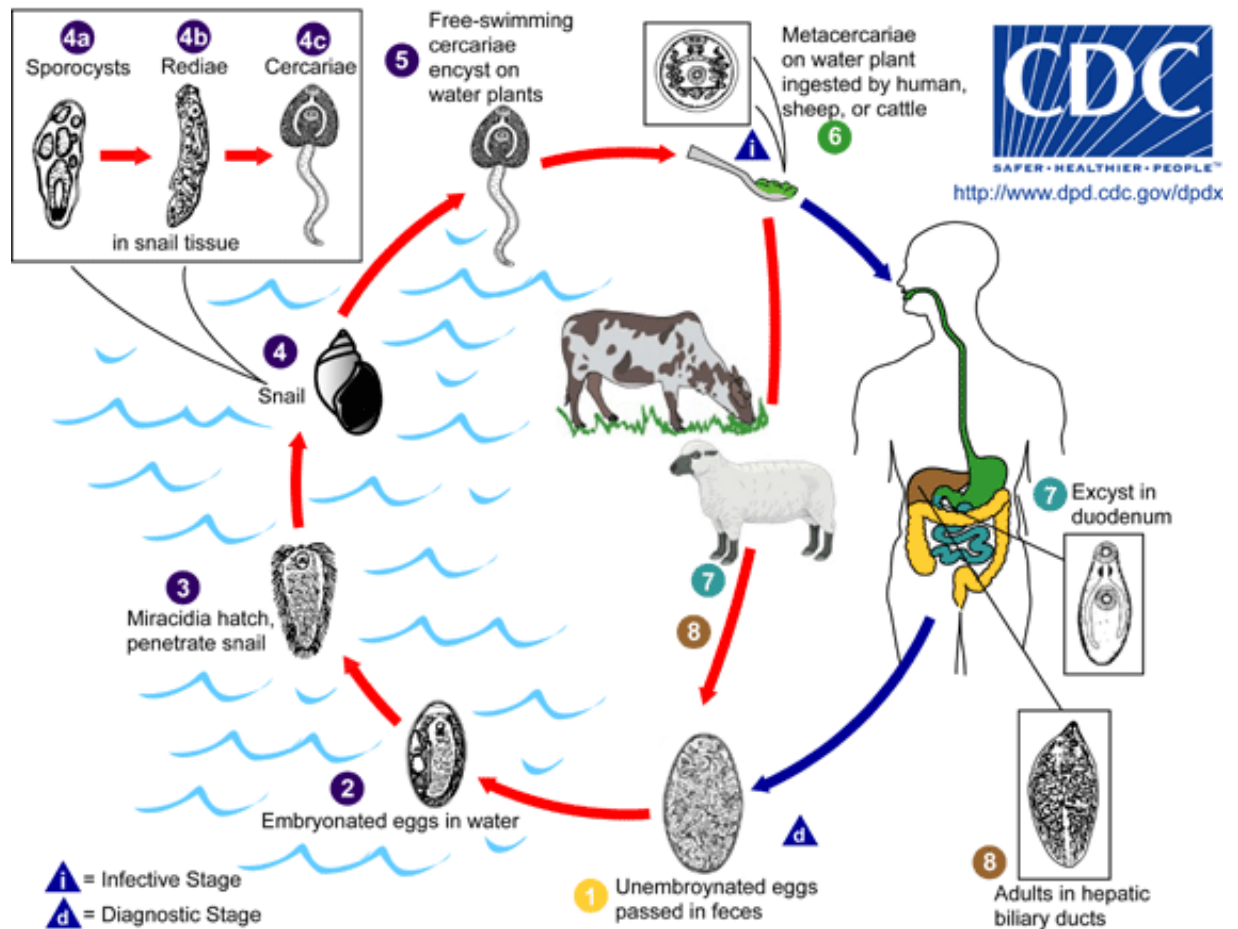


Fig. 1: The life cycle of *F. hepatica*.
 (http://dpd.cdc.gov/dpdx/HTML/ImageLibrary/Fascioliasis_il.htm)

The life cycles of *F. buski* and *F. magna* are very similar to *F. hepatica*, but there are differences of the localization in the definitive hosts, the sizes, forms and colours of eggs or adult flukes. The adults of *F. hepatica* parasitize the biliary tract (Eckert *et al.*, 2008). Generally the adults of *F. buski* parasitize the small intestine, but in heavy infections they were also found in the stomach or intestine regions (Roberts *et al.*, 2009). Juveniles of *F. magna* burrow through the liver, whereas adults encapsulate in pairs or in higher numbers in the liver parenchyma [(Foreyt *et al.* 1976) cit. in Spakulova, 2003].

1.1.1.3. Occurrence and distribution

F. hepatica was first described in 1379 by Jean de Brie, known as the common liver fluke or the sheep liver fluke, in animals; in 1600, *F. hepatica* was also discovered in humans (Ash and Oriel, 2007). *F. hepatica* occurs worldwide, especially in sheep or cattle. Not only domestic ruminant species become infected, but also other animal species for example horses, pigs and humans (Schnieder, 2000). *F. hepatica* prevails in temperate zones and is dominant in Europe, the Americas and Oceania (Mas-Coma *et al.*, 2005). At the moment *F. buski* is endemic distributed in Asia and on the Indian subcontinent (Mehlhorn, 2008) and parasitizes pigs and humans (Cheng, 1986). The great American liver fluke, *F. magna*, is widely spread in North America and parasitizes cervids, especially white-tailed deer and wapitis. The definitive hosts in Europe are red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) (Erhardová-Kotrlá and Kotrly, 1968). Cattle, sheep and other animal species can also be infected. But they act as accidental hosts, in which *F. magna* usually will not become sexually mature (Eckert, 2008). In North America, *F. magna* is mainly found in the region of the Great Lakes, the Gulf coast, the lower part of the Mississippi, the South Atlantic coast, North Pacific coast, Rocky mountains, North Quebec and Labrador (Pybus, 2001). During the second half of the 19th century *F. magna* was introduced to Europe by animal transports of Wapiti (Bassi, 1875). First records of *F. magna* were found in deer in a zoo in Turin, Italy (Bassi, 1875). The parasite was also verified in the 20th century in the Czech Republic (Ullrich, 1930), Germany (Salomon, 1932) and Poland (Slusarski, 1955). *F. magna* was also recorded from Slovakia (Rajsky *et al.*, 1994), Hungary (Majoros and Sztojkov, 1994) and Croatia (Marinculic *et al.*, 2002; Janicki *et al.*, 2005). The present days, *F. magna* occurs regional enzootic in red and roe deer in the Czech Republic, Slovakia, Hungary, Croatia and Austria (Eckert *et al.*, 2008). In Austria, the first infected fallow deer was found in a game reserve close to the Danube in Lower Austria (Pfeiffer, 1983). In the year 2000, the first finding in the wild was registered in red deer (Winkelmayer and Prosl, 2001; Ursprung and Prosl, 2011). In the past years, the parasite distributed in free-living red and roe deer in the floodplains of the Danube, especially in the regions of Fischamend, Regelsbrunn, Orth and Mannswörth (Winkelmayer and Prosl 2001; Ursprung, 2002; Sattmann *et al.*, 2014). The first record of the fluke in the later environments in fallow deer (*Dama dama*) was proofed by Ursprung (2013).

1.1.1.4. Medical relevance

Fascioliosis is an infection caused among others by *F. magna* and *F. hepatica* (Connor *et al.*, 1997). The disease belongs to the group of foodborne trematodes infections and is a zoonosis. A zoonosis is an animal infection that also may be transmitted to humans. Fascioliosis is an important veterinary disease and has an extensive economic effect on the farming industry (Del Campillo and Vazquez, 1999). The giant liver fluke has a high pathogenic potential in cervids and is continuously spreading, especially in Europe. Infections with *F. magna* occur originally in North America, but meanwhile also in European countries, northern Asia and Africa (Mas-Coma, 2004). The cervids are mainly affected by the parasite's migration within the tissue. This may have fatal effects and lead to strong liver damage or even to the death of the host (Pybus, 2001). The livers of red deer show chronic pathologies and, depending on the parasite load, the animals become weakened or die spontaneously without marked symptoms. Infections of accidental hosts, for example goats, sheep and even roe deer, can be lethal (Foreyt, 1996; Erhardova-Kotrla and Kotrly, 1968). Human fascioliosis (caused by *F. hepatica*) has always been viewed as a secondary disease, but the public health importance has increased since the 1980s (Malek, 1980). Between the period of 1970 to 1990 a high number of new infections was recognized: 2594 infected persons in 42 countries distributed all over the world (Mas-Coma *et al.*, 1998). The WHO (2012) states that human infestations occur occasionally but are increasingly reported from Europe, the Americas and Oceania and from Africa. The WHO estimates that about 2.4 million people are infected in more than 70 countries worldwide, with several million at risk. (http://www.who.int/foodborne_trematode_infections/fascioliasis/en/). Potential

infection sources for humans are wild herbs, vegetable and watercress (*Nasturtium officinale*), grown in wet or occasionally flooded areas. But also any other plant species can be an infection source, depending on the different countries and different dietary habits, for example in France dandelion (*Taraxacum spp.*) or spearmint (*Mentha viridis*) (Mas-Coma *et al.*, 1998). After the larvae are ingested with contaminated food or water, a symptomless incubation period starts, lasting for a few days or months. This is followed by two different stages, an acute and a chronic clinical phase. The acute phase lasts between one to three months and begins when the immature worms penetrate the intestinal wall and the peritoneum (Aksoy *et al.*, 2005). From here, the parasite starts to migrate through the liver parenchyma towards the biliary ducts. The liver cells are

damaged by the invasion through the parenchyma, which causes intense internal bleeding. The symptoms are fever, pain in the right hypochondrium, hepatomegaly, hypergammaglobulinaemia and eosinophilia (Aksoy *et al.*, 2005). The chronic phase begins when the worms reach the bile ducts, where they become sexually mature and start producing eggs. The symptoms are intermittently pain, jaundice and anaemia. Pancreatitis and gallstones may also occur (http://www.who.int/foodborne_trematode_infections/fascioliasis/en/). Eosinophilia can be detected as well (Hadden *et al.*, 1967; Jones *et al.*, 1977). For animals, a treatment with Fasinex® (Novartis Animal Health Inc.), a 10% suspension of triclabendazole is recommended. Triclabendazole, a benzimidazole, is an antihelminthic and is active against the immature and the adult stages of *Fasciola* spp. (Novartis: <http://www.ah.novartis.de/platform/content/element/1682/FasinexFachinfo.pdf>). The appropriate recommended dose is 10 mg/kg body weight (http://www.who.int/foodborne_trematode_infections/fascioliasis/fascioliasis_diagnosis/en/index.html).

F. buski is the causative agent of the disease fasciolopsiosis. The infection of human and pigs is acquired by eating encysted metacercariae on aquatic plants like raw water spinach (<http://www.cdc.gov/parasites/fasciolopsis/faqs.html>). *F. buski* is endemic in Asia, especially in China, and the Indian subcontinent. The disease occurs frequently in countries lacking proper sanitation systems (Keiser *et al.*, 2009). In most cases, the infections are asymptomatic and light, but there are also heavier infections with diarrhea, abdominal pain, fever, ascites and intestinal obstruction. Praziquantel is the drug of choice (<http://www.dpd.cdc.gov/dpdx/HTML/Fasciolopsiasis.htm>). Tab. 1 summarizes the main facts about *F. magna*, *F. hepatica* and *F. buski* and their linked diseases.

Tab. 1: Distribution, niche, intermediate- and definitive host and symptoms of *F. buski*, *F. magna* and *F. hepatica*. This overview is complied from: Eckert *et al.*, 2008 ; Keiser *et al.*, 2009)

	Distribution	Niche	Intermediate host	Definitive host	Symptoms
<i>Fascioloides magna</i>	USA, Europe	Liver parenchyma	Freshwater snail	Herbivores, Ungulates, mainly Cervids; not humans!	No gain in weight, reduced milk production, curtailed breeding
<i>Fasciola hepatica</i>	World-wide	Bile ducts	Freshwater snail	Sheep, cattle, deer, pigs, humans	“Fasciolosis”
<i>Fasciolopsis buski</i>	Asia	Small intestine	Freshwater snail	Humans, pigs	Ulceration, abcess in the small intestine

1.1.2. Schistosomatidae

The Schistosomatidae are also an important member of the class Trematoda. These digenean flukes parasitize birds, mammals and crocodiles. The family of the Schistosomatidae includes 14 recognized genera and about 100 species (Khalil, 2002). Within the family of Schistosomatidae the genus *Schistosoma* contains species that parasitize humans. For instance, *Schistosoma mansoni* causes human intestinal schistosomiasis and is widespread in Africa, South America and also in the Caribbean (Lockyer *et al.*, 2003).

1.1.2.1. Classification and morphology

The phylogenetic studies of the Schistosomatidae are mainly based on nuclear (28S and ITS rDNA) and mitochondrial (cox1) markers (Jouet *et al.*, 2010). There are also a lot of data and theories about the phylogeny of the family of the Schistosomatidae. Based on the data of the 18S rDNA sequence, *Orientobilharzia* and *Schistosoma*, both infecting only mammals, form a monophyletic clade. The second clade is formed by the other schistosomatid taxa primarily infecting avian hosts, such as *C. olor*, *M. merganser*, *A. clypeata* (Jouet *et al.*, 2009) or *A. platyrhynchos* and *A. fuligula* (Rudolfová *et al.*, 2002). The remaining bird and mammal schistosomes represent three further clades: *Schistosomatium* and *Heterobilharzia*; *Dendritobilharzia*, *Gigantobilharzia*, *Trichobilharzia* and *Bilharziella*; and *Ornithobilharzia* and *Austrobilharzia* (Lockyer *et al.*, 2003). *Bilharziella* is a small genus that contains only one species in Europe: *Bilharziella polonica*. The largest genus of the family Schistosomatidae represents the genus *Trichobilharzia* La Valette, 1855 (Kolárová *et al.*, 2013). Until now five species have been found in Europe: *T. regenti* (Horák *et al.*, 1998) developing usually in *Radix peregra*, *T. franki* Müller & Kimmig, 1994 developing in *Radix auricularia*, *T. szidati* Neuhaus, 1952 developing in *Lymnaea stagnalis* (Jouet *et al.*, 2010), *T. salmanticensis* (Simon-Martin and Simon-Vicente, 1999) and *T. mergi* developing in *R. balthica* Linnaeus, 1758 (Kolarova *et al.*, 2013). In the literature *T. ocellata* La Valette, 1855 (Brumpt, 1931) is mentioned as a synonym for *T. szidati* (Rudolfová *et al.*, 2005). The ‘bird schistosomes’ usually parasitize birds of the family Anatidae, from which they have their name. They can be classified into two groups according to the localization of the adult worms in the natural final hosts (Horák *et al.*, 2002). The visceral schistosomes are located in the venous system of the intestinal and hepatic sphere. They

migrate via the blood vessels within the final host. The nasal schistosomes live in the nasal cavities of their host. The nasal schistosomes migrate through the nervous system of the final host before they reach their final localization, where they mature into adult worms (Hrádková and Horák, 2002; Chanová and Horák, 2007). But the cercariae are also able to penetrate human skin. However, they cannot complete their life cycle in these accident hosts and die shortly after the penetration (Horák and Kolárová, 2005). The genus includes visceral (*T. szidati*, *T. franki*, *T. salmanticensis*, *T. mergi*) and the nasal (*T. regenti*) forms. According to phylogenetic analyses, the visceral *T. franki* is more related to the nasal *T. regenti*, than to the visceral *T. szidati* (Dvorák *et al.*, 2002).

1.1.2.2. Life cycle

The Schistosomatidae have a two-host life cycle, with water snails and birds as intermediate and final hosts, respectively. The life cycle of the different species of *Trichobilarzia* spp. is rather similar with the exception of *T. regenti*. Details of the life cycle are given in Fig. 2. In brief, the matured eggs, containing fully developed miracidiae, leave the host with the feces. Once in the water, the eggs liberate miracidiae. With help of cilia, the miracidiae swim around searching for a molluscan intermediate host. As soon as they have found a proper intermediate host, they penetrate it. In the intermediate host each miracidium develops into a mother sporocyst, which produces daughter sporocysts. The daughter sporocysts migrate into the hepatopancreas where further development continues. Finally, free-swimming cercariae with a forked tail are released. After leaving the intermediate host they start searching for a suitable final bird host such as ducks. The cercariae have a chemotrophic reaction to secretions from the skin and are not as host-specific as other types of schistosomes. They find and recognize their bird hosts by different phases of host-finding behaviour, e. g. optical, tactile and olfactorial (Feiler and Haas, 1988a,b ; Haas and Van de Roemer, 1998), but they cannot differentiate between ducks, humans and other endotherms. Therefore they also penetrate mammals including humans contacting the contaminated water of lakes. The penetration causes an itching dermatitis, so-called cercarial dermatitis (Hertel *et al.*, 2002). In birds, the cercariae penetrate the skin while dropping off their tail. In the skin of their definitive host, the cercariae begin to transform into schistosomula and then start to migrate to the blood system of visceral organs to mature into adults and deposit their eggs. In comparison to the majority of bird schistosomes, the schistosomula of *T.*

regenti migrate from the skin via the spinal chord and brain to the nasals, where they lay eggs (Horák *et al.*, 1999). Miracidia are able to hatch from the eggs directly within the host tissue, the nasal mucosa, waiting for contact with water (Horák *et al.*, 1999). The development of the worms seems to occur in the nervous system. In mammals, the parasite dies in the CNS without mating and offspring production (Dvorák *et al.*, 2002).

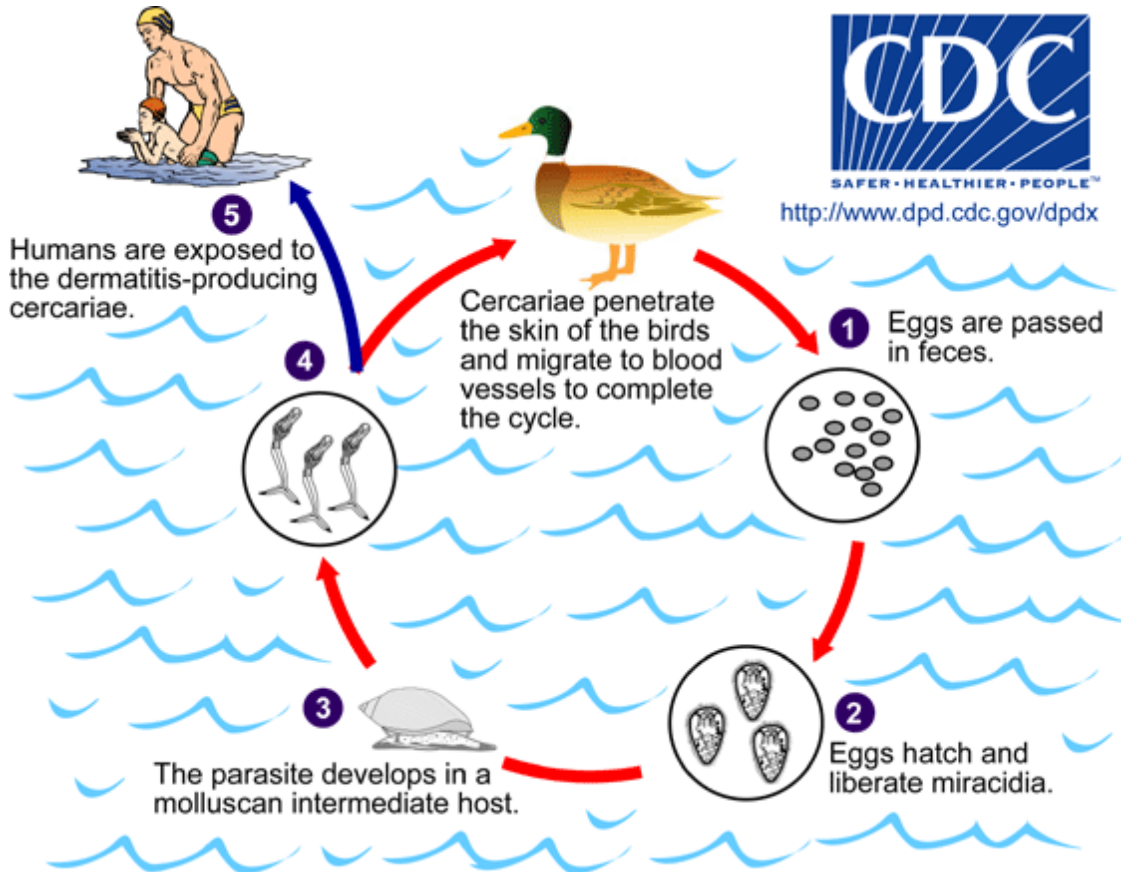


Fig. 2: The life cycle of *Trichobilharzia* spp.
(<http://www.cdc.gov/parasites/swimmersitch/biology.html>).

1.1.2.3. Occurrence and distribution

As mentioned before, until now, five species of the genus *Trichobilharzia* are known to occur in Europe: *T. franki*, *T. regenti*, *T. szidati*, *T. salmaticensis* and *T. mergi*. *T. franki* has been reported in many countries in Europe, such as in the Czech Republic (Kolárová *et al.*, 1997; Rudolfová *et al.*, 2005), Germany (Brant and Loker, 2009) Switzerland (Picard and Jousson, 2001), France (Ferté *et al.*, 2005; Jouet *et al.*, 2009) Iceland (Skirnisson and Kolárová, 2008; Skirnisson *et al.*, 2009; Aldhoun *et al.*, 2009), Russia (Semyenova *et al.*, 2005), Belarus (Chrisanfova *et al.*, 2009), Poland (Rudolfová *et al.*, 2005) and Finland (Aldhoun *et al.*, 2009). Four more *Trichobilharzia* species have

been discovered in North America: *T. physellae* Talbot, 1936 (McMullen and Beaver, 1945) developing in *Physa parkeri*, *T. querquedulae* McLeod, 1937 developing experimentally in *Physa acuta*, *T. stagnicola* Talbot, 1936 (McMullen and Beaver, 1945) developing in *Stagnicola emarginata* and *T. brantae* (Farr and Blankemeyer, 1956) developing in *Gyraulus parvus* (Jouet *et al.*, 2010). According to the latest phylogenetic DNA analyses *T. brantae* is not a part of the genus *Trichobilharzia* (Brant and Loker, 2009). The occurrence of *Trichobilharzia* spp. depends on various conditions promoting the parasite's transmission. A complex interaction of the parasites and their specific intermediate and definitive hosts is required at the same place. Cercariae of bird schistosomes may occur in lakes all over the world, because their larval development takes place in freshwater snails. In a temperate climate, cercarial dermatitis occurs seasonally, mainly during warmer months when both, the release of cercariae from snail intermediate hosts and the number of people swimming in natural waters, reach highest levels (Appleton and Lethbridge, 1979).

1.1.2.4. Medical relevance

The members of the family Schistosomatidae can cause schistosomiasis or bilharziosis, a tropical parasitic disease mainly found in developing countries in South America, Asia and Africa. The main disease-causing species are *S. mansoni*, *S. japonicum* and *S. haematobium* (Gryseels *et al.*, 2006). After the first infection, most people show no symptoms. But after a few days, they may develop an itchy skin and after 1-2 months the symptoms are similar to a feverish infection (<http://www.cdc.gov/parasites/schistosomiasis/disease.html>). The diagnostic standard is a microscopic demonstration of eggs in excreta. The drug praziquantel is the gold standard for treatment; vaccines are not yet available. The acylated quinoline-pyrazine is active against all schistosome's species and acts within 1h of ingestion. The worms become paralysed and their tegument gets destroyed (Gryseels *et al.*, 2006). Without a treatment, the schistosomiasis develops into a long lasting infection. The symptoms are abdominal pain, blood in the stool or urine, pain while passing urine and an enlarged liver (<http://www.cdc.gov/parasites/schistosomiasis/disease.html>).

In humans, flukes of the family Schistosomatidae can also cause cercarial dermatitis - species of the genus *Schistosoma* as well as species of other genera like *Bilharziella* and *Trichobilharzia*. While searching their definitive host the cercariae respond to swirled

up water, sudden shadow, temperature differences and also to different body fats such as cholesterol and ceramid (Feiler and Haas, 1988a,b; Haas and van de Roemer, 1998). Humans and water birds both use the same water and fulfil the conditions for a proper definitive host. By mistake, they penetrate the human skin (Allgöwer, 1990a). In 1887, Fukji was the first describing the clinical symptoms of cercarial dermatitis (Oda, 1973). After repeated skin penetrations of *Trichobilharzia* spp. the human skin shows strong, maculo-papulo-vesicular skin eruption accompanied by intensive itching. The human immune system causes a dermal inflammatory response, depending on the degree of hypersensitivity induced by previous exposures (Hoeffler, 1974). Within minutes to days after contact with contaminated water, symptoms like small blisters or reddish pimples and burning or itching skin appear. These skin affecting syndromes can also appear in various animals, for example in rabbits and dogs (Augustine and Weller, 1949; Olivier, 1953; Sattmann *et al.*, 2004). To reduce the risk of getting the cercarial dermatitis, swimming in plant-rich waters where snails commonly found should be avoided. Towelling dry or showering immediately after leaving water can help to reduce potential infections/ entry of the cercariae (<http://www.cdc.gov/parasites/swimmersitch/faqs.html>).

1.2. Intermediate hosts

Digeneans use one or more intermediate hosts for their developmental stages and asexual reproduction. The intermediate hosts can contribute to the distribution of the parasite and also help to find the proper definitive host. Numerous members within the families Lymnaeidae or Planorbidae are intermediate hosts for veterinary and medical highly important Digenea. The parasite-host specificity depends on finding and recognizing the proper intermediate hosts, but also the compatibility, resistance and immunity, regarding the parasite, respectively (Yoshino and Vasta, 1996, Bargues *et al.*, 2001).

1.2.1. Lymnaeidae

The freshwater snail family Lymnaeidae belongs to the order Basommatophora and to the class Gastropoda. Within the subfamily Lymnaeinae there are several important genera, namely *Lymnaea* Lamarck, 1799, *Galba* Schrank, 1803 and *Radix* Montford, 1810. The species *G. truncatula* lives amphibious and is a small freshwater snail. The shell's height reaches about 5-10 mm and the maximum length is about 12.0 mm (Bargues *et al.*, 2012). The shell is cone-shaped, right-coiled and horn-coloured, the whorls are stepped and the columella is folded (Bargues *et al.*, 2012). The shell appears pale because of the large unpigmented whitish spots on the mantle roof. *G. truncatula* is found all over the world from Transcaucasus to Ukraine, West- and Eastsiberia, Central Asia (Zhadin, 1965; Schütt, 1983) up to Europe and also to North America (Glöer and Meier-Brook, 1998; Pfleger, 1999; Glöer, 2002). The snail was also found in a very high altitude such as in Altiplano, in northern Bolivia (Bargues *et al.*, 2012) and was dispersed to many regions in the world. The snail is also widespread in Austria such as in the 'Nationalpark Donau-Auen' and adjoining zones (Haider *et al.*, 2012). *G. truncatula* inhabits both temporary and permanent freshwater habitats. The snail prefers shallow well-oxygenated water in ponds, lakes, rivers or sandbanks (Kendall, 1949; Chapuis *et al.*, 2007). Mostly it occurs on the edge between water and land, and on muddy sandbanks. *G. truncatula* can survive long dry periods from 6 weeks up to 4.5 months in an aestivated stage in the mud. This is caused by its high ability to aestivate during drought conditions (Kendall, 1949). Usually, *G. truncatula* subsists on algae and parts of plants (Beran, 1998). The snail has a life span of up to two years (Jackiewicz, 2000). The snails, which self fertilize in the majority of the cases (Trouvé *et al.*, 2003),

lay their eggs in clusters in highly humid places (Kendall *et al.*, 1953). The egg clutches are round to oval and can contain 10 to 20 eggs (Bargues *et al.*, 2012). *G. truncatula* is semelpar (Glöer *et al.*, 2002). Twice a year, in spring and autumn, there are ovipositions from two different generations (Morel-Vareille, 1973; Fretter and Peake, 1975). The autumn born generation hibernates and supports the next breeding season in the spring. The breeding season of the spring generation starts usually in autumn (Mekroud *et al.*, 2002). Until the seventh week they grow about 1 mm per week, afterwards the growth slows down. In general, fully-grown snails can reach a shell height of about 12 mm (Morel-Vareille, 1973). Sexual maturity is reached with the age of two months; by that time they will have reached a height of 4 mm (Smith, 1981). *G. truncatula* serves as an intermediate host for different species such as *F. hepatica*, *F. magna* and also *Paramphistomum daubneyi* (Dreyfuss *et al.*, 2005).

The species *L. stagnalis*, better known as the great pond snail, belongs to the large air-breathing freshwater snails of the family Lymnaeidae. The height of an adult shell is about 45-60 mm, the colour varies from yellow-brown to dark-brown and has a pointed spire. The skin of *L. stagnalis* is dark brown or grey (Glöer *et al.*, 2002). The central nervous system (CNS) is relatively simple and consists of about 20,000 neurons. The CNS of *L. stagnalis* serves as a model system for the investigation of neuronal regeneration, synaptic plasticity or adaptive response to hypoxic stress (Feng *et al.*, 2009). *L. stagnalis* is widely distributed over Europe for example in the Czech Republic (Jurickova *et al.*, 2001), Germany (Glöer *et al.*, 2002) and also in Austria (Reischütz, 1981). The snail lives only in freshwater, preferring slowly running rivers or standing ponds. *L. stagnalis* is a simultaneously hermaphroditic species, but prefers sexual mating (Cain, 1956). The egg-deposition is in jelly-like capsules on the bottom side of aquatic plants. Three months after hatching, the young snails become sexually mature (Grabow, 2000), whereby the sexual organs of the males mature two weeks earlier than the sexual organs of the females (Duncan, 1975). The reproductive period lasts from the end of April until October (Heitkamp, 1982). Their life span is up to two years (Glöer, 2002). *L. stagnalis* absorbs air via floating upside-down on the surface, but also from the water through the skin. Because of this feature, it is possible for the snail to stay under water for several months. In case of desiccation it survives in the mud. *L. stagnalis* serves as an intermediate host for *Echinostoma revolutum*, for *T. szidati* and several other Digenea (Soldanova *et al.*, 2010).

1.2.2. Planorbidae

The Planorbidae Rafinesque, 1815, also named ramshorn snails, are a freshwater snail family of the class Gastropoda. The species within this family are spread over all continents as well as several islands. A special focus is given here on two species within this family, namely *Planorbis planorbis* Linnaeus, 1758 and *Planorbarius corneus* Linnaeus, 1758. *P. planorbis* has a left-coiled shell with a width of 15 to 20 mm and a wide up to 3.5 mm. It is almost planispiral, the shell is flat-coiled, bright-brown, transparent and very robust. The body itself is coloured from light to dark grey. The snail has two long, round tentacles, which are red-coloured. *P. planorbis* has red-coloured blood, containing haemoglobin. Due to this, *P. planorbis* is able to survive under oxygen-poor conditions. For air breathing, the snail has to come up to the surface. It prefers slowly running water, full of water plants with a muddy bottom, near the shoreline (Pfleger, 1984). This species is widely distributed throughout the Palearctic region, Norway, across Europe and throughout parts of North Africa (Fechter and Falkner, 1990; Glöer 2002). *P. planorbis* is a hermaphrodite. After fertilization, the snail produces capsules of eggs, which develop in about 11 to 14 days. *P. planorbis* has a life span from one up to four years (Glöer, 2002). It serves as an intermediate host for a number of trematodes, for example *Echinoparyphium pseudorecurvatum* (Faltynkova *et al.* 2008).

Planorbarius corneus Linnaeus, 1758 is a planorbid gastropod with a left-coiled shell as well, that is coloured in dark-red up to brown or grey. The body of the snail is dark brown and the foot is as long as the shell's diameter. *P. corneus* is widely distributed from Europe to Eastern Siberia, also in Norway, Sweden, Finland, Russia (Vinarski *et al.*, 2007), Poland, the Czech Republic and Austria (Glöer, 2002). The snail prefers a habitat with still or only slowly moving water as well as floodplains or lakes (Angelov, 2000). *P. corneus* feeds on different kinds of algae, seaweed, detritus and carrion and likes a high level of dissolved calcium in the water (Janus, 1965; Grabow, 2000). *P. corneus* is also hermaphroditic. The egg deposition takes place in summer, when the temperature of the water is at least 12°C (Frömming, 1954). The eggs, appearing as a flat, jelly-like clump, are attached to the bottom side of leaves or of stones. Under optimal conditions the snail grows up to 1 mm per week until it becomes sexual mature, afterwards the growth declines. The snail hibernates in mud and its life span lasts up to

three years (Glöer, 2002). *P. corneus* can also serve as an intermediate host for different kinds of trematodes, such as *Bilharziella polonica* (Dvorak *et al.*, 1999).

1.3. Previous studies in Austria

The so-called Neobiota are animals or plants that were released to new regions. The species are characterized by their relatively rapid spread in new environments, causing ecological or economic damage. Most of the introduced species do not cause significant harm or threat in their novel territories. But there are species having negative effects on the health of animals and humans (Eliás, 1997). Also in Austria introduced species appeared within the last century, for example *F. magna* (Pfeiffer, 1983). At the end of the 19th century, the large American liver fluke *F. magna* has been repeatedly introduced to Europe by imported American cervids. In Austria, *F. magna* was detected for the first time in fallow deer in a game reserve (Pfeiffer, 1983). In autumn 2000, infected cervids were found in the Danube floodplain forest area of 'Fischamend' for the first time in the wilderness. In the following years, the parasite spread in free-living red and roe deer in the floodplains in the east of Vienna (Winkelmayer and Prosl, 2001; Ursprung 2002). Already one year after the first discovery of *F. magna* in the wild, a monitoring and control programme was started in the floodplains of the Danube. Free-living deer was treated with Fasinex® (Novartis Animal Health Inc.). The drug was admixed into the winterfeed, thus administering 10-15 mg triclabendazol per kg body weight within a period of six days (Ursprung *et al.*, 2005). From 2001 to 2005, the treatment was performed 3-4 times per year. Since 2006 it was executed twice a year (Ursprung *et al.*, 2011). A very low prevalence (0.03%) of *F. magna* in *G. truncatula* was recognized in the year 2004/2005, when the first screening was conducted (Sattmann and Hörweg, 2006). But a complete eradication of *F. magna* could not be achieved. In the year 2006, the region around 'Orth' in the floodplains of the Danube in the east of Vienna was identified as a risk area based on a GIS risk analysis (Reckendorfer and Groiss, 2006). Additionally a shift to the northern side was noticed and the prevalence of *F. magna* infected definitive hosts (red deer) increased up to 72% (Ursprung and Prosl, 2011). After the observed relapse, a study in order to analyse the infection dynamics in the intermediate host was initiated. In a total of 3,444 collected *G. truncatula*, *F. magna* was detected with a prevalence of 0.23% (Haider *et al.*, 2012). Additionally, digenean parasites were detected with a prevalence of 2.41% (1.83%

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Paramphistomoidea; 0.46% Echinostomatoidea; 0.09% Strigeida; 0.06% Plagiorchiida) (Haider *et al.*, 2012). In these areas, it is very important to execute further monitoring to keep introduced species like *F. magna* under control. It is also important to recognize new species as quickly as possible and take measures against them. For most parasites it is very easy to establish in new areas as long as the intermediate host and the final host exist (Sattmann *et al.*, 2014). An example could be *F. buski*, which is at the moment endemic in Asia, but the intermediate hosts, for example *Lymnaea* spp., and the final host, pigs and humans, exist in a lot of countries (Keiser, 2009).

Another focus was directed onto the occurrence and distribution of local species, for example *Trichobilharzia* spp. After accumulated occurrence of cercarial dermatitis in the year 1967, the cercariae of the species *B. polonica* and *T. szidati* have been proven at Lake 'Neusiedl' for the first time (Graefe, 1971; Graefe *et al.*, 1973). Since that in all federal states of Austria – except for Vorarlberg – continually cases of cercarial dermatitis were observed. In all examined cases the cercariae were identified based on morphological characteristics, namely furcocercariae of the species *B. polonica* or *T. szidati* (Auer and Aspöck, 1995; Sattmann *et al.*, 1997). However, the knowledge of these parasites was still incomplete in Austria, so a bilateral research project with the Czech Republic was started (Dvorák *et al.*, 1999). Five genera of schistosomes have been described in the Czech Republic (Vojtek, 1981 [cit. in Dvorák *et al.*, 1999]). Due to the geographical adjacency to the Czech Republic, the situation was assumed to be similar in Austria. To verify this assumption, a total of 5,073 of 14 different freshwater snail species were collected in 20 localities in the surroundings of Vienna, Salzburg and Illmitz (Dvorák *et al.*, 1999). But only two species of Schistosomatidae in three species of snails were found: seven *L. stagnalis* infected with *T. szidati* were found in two different ponds (First pond: n=96, prevalence 5.2%; Second pond: n = 32, prevalence 6.3%). Twice, on different days *Trichobilharzia* sp. were found in *Radix peregra ovata* (n=45, prevalence 2.2%; n= 33, prevalence 6.1%), and *B. polonica* in *P. corneus* (n= 26, prevalence 3.8%) (Dvorák *et al.*, 1999). Further, in the year 2003, cases of cercarial dermatitis have been noticed in 'Neue Donau' [(Steinspornbrücke, Konecny personal communication) cit. in Sattmann *et al.*, 2004] in Vienna, 'Offensee' in Upper Austria and some swimming ponds south of Vienna (Sattmann *et al.* 2004). The authors only found one individual of *Radix ovata* Draparnaud, 1805 that shed living cercariae (Sattmann *et al.*, 2004). Taken together, in Austria different snail species have been

identified as intermediate hosts for schistosomatid cercariae. In a previous study, they were able to prove *T. szidati* in Austria. They infected ducks with cercariae of *T. szidati* out of *L. stagnalis*, found in the Wienerwald and determined the adult schistosomes (Dvorák *et al.*, 1999). Additionally, schistosomatid cercariae were found in other snail intermediate hosts, like *Stagnicola turricola* Held 1836, *Stagnicola* sp. and *Aplexa hypnorum* (Hörweg & Sattmann, unpublished [cit. in Sattmann *et al.*, 2004]). Furthermore, in Carinthia, morphologically similar cercariae were found in *Gyraulus parvus* Say 1817, a snail-species introduced from North America, (Mildner and Sattmann, 1998 [cit. in Sattmann *et al.*, 2004]). In all previous studies, cercariae were detected repeatedly in different freshwater snail species. A complete list of all schistosomatid cercariae and the respective host snails found in Austria, is given in Tab. 2. Until today, the species spectrum in Austria is still very small. One reason for this could be that morphological determination of cercariae is difficult. Therefore, it is important to combine morphological and molecular biological methods to determine the true species spectrum of schistosomatid parasites in Austria.

Tab. 2: Detected schistosomatid cercariae in snails in Austria (Sattmann *et al.*, 2004)

Snail species	Schistosomatid species
Family Lymnaeidae	
<i>Lymnaea stagnalis</i>	<i>Trichobilharzia</i> sp., <i>Trichobilharzia szidati</i>
<i>Stagnicola turricola</i>	<i>Trichobilharzia</i> sp.
<i>Stagnicola</i> sp.	<i>Trichobilharzia</i> sp.
<i>Radix auricularia</i>	<i>Trichobilharzia</i> sp.
<i>Radix ovata</i>	<i>Trichobilharzia</i> sp.
Family Physidae	
<i>Aplexa hypnorum</i>	<i>Schistosomatidae</i> gen.sp.
Family Planorbidae	
<i>Planorbarius corneus</i>	<i>Bilharziella polonica</i>
<i>Gyraulus parvus</i>	<i>Schistosomatidae</i> gen.sp.

1.4. Aims of the study

The current study, which was initiated by the ‘Landesjagdverband Niederösterreich’ and the Natural History Museum Vienna, was conducted in the floodplains of the rivers Bruck/Leitha and the surrounding areas, in the east of Vienna. It was planned to build a wildlife crossing over the highway A4 at the level of the chapel ‘Adelsberg’, which is close to ‘Arbesthal’. The construction has already started (Fig. 3).



Fig. 3: Construction of the wildlife crossing

With the construction of the wildlife crossing, the area between the river Danube and the river Leitha will be connected for several game species including deer. Wildlife crossings allow animals on the one hand to cross human-made barriers safely, thus to mitigate, but on the other hand also parasites to migrate with the animals.

The first aim of the current study was to proof the occurrence of *G. truncatula*, *L. stagnalis*, *P. planorbis* and *P. corneus*. These freshwater snails serve as intermediate host for digenean trematodes such as *F. magna* and *F. hepatica*, developing in *G. truncatula*, *T. szidati*, developing in *L. stagnalis* and *P. planorbis*, or *B. polonica*, developing in *P. corneus*. For this purpose, a monitoring of the intermediate hosts was performed in the area between the floodplains of the River Leitha near to ‘Götzendorf an der Leitha’ (Lower Austria) to ‘Pötzneusiedl’ (Burgenland). The locations were chosen according to the following criteria: drinking water points of deer, for example periodic flooding meadows, and possible migration points.

A second aim was to determine the infection rates of these snails with digenean trematodes to reveal the digenean diversity in general. In particular the occurrence of Fasciolids and Schistosomatids has been analysed. In order to assess the trematodes in

the intermediate host, the snails were examined by microscopy. As a morphological differentiation of closely related species is often not possible, biomolecular techniques were included into the study. After performing a PCR, the positive amplicons were sequenced and compared to one another and to already existing trematode sequences in GenBank by multiple alignments.

2. Material and methods

From March 2012 until November 2012 a total of 1,184 snails (781 *G. truncatula*, 339 *L. stagnalis*, 63 *P. planorbis* and 1 *P. corneus*) were collected at 29 locations along or near the river Leitha and 2 further locations in collaboration with my colleague Anna-Sophia Feix and under supervision of Helmut Sattmann and Christoph Hörweg. Altogether, 781 individuals of *G. truncatula*, 66 individuals of *L. stagnalis*, 3 individuals of *P. planorbis* and 1 individual of *P. corneus* were checked microscopically for the presence of parasites. Out of these, 53 individuals of *G. truncatula*, 66 individuals of *L. stagnalis*, 3 individuals of *P. planorbis* and 1 individual of *P. corneus* were presumably positive for trematodes. The found digenean trematode stages were tested by PCR. By subsequent sequencing of the PCR products and alignments with reference sequences species identification was possible in most cases.

2.1. Collection of the intermediate host snail

Altogether 1,184 freshwater snails were collected in the floodplains of the river Leitha, between ‘Götzendorf’ and ‘Pötzneusiedl’. The 31 locations associated with geographical coordinates are shown in Tab. 3.

Tab. 3: Sampling sites with geographical details. The 31 sampled locations are listed with number, name and geographical position.

No.	Location	N°	E°
1	Leitha A4 Leithabrücke Bruck-Ost 1	48,02925	16,8273
2	Leitha A4 Leithabrücke Bruck-Ost 2	48,02826	16,82503
3	Leithazwickl	48,02846	16,82382
4	Pachfurth Auwinkel "Altarm" bei Weinberg	48,03744	16,83719
5	Pachfurth Auwinkel	48,03716	16,8365
6	Hollern	48,07316	16,89389
7	Eco-Plus Park 1	48,02856	16,80366
8	Eco-Plus Park 2	48,02956	16,80861
9	Gärtnerstrasse	48,01466	16,76702
10	Gärtnerstrasse 2	48,01399	16,76542
11	Steinbach	48,00847	16,75155
12	Wilfleinsdorf	48,01188	16,7167
13	Sarasdorf Leithakanal	48,01986	16,6722
14	Sarasdorf Brücke	48,02010	16,67228
15	Trautmannsdorf	48,02010	16,6337
16	Pötzneusiedl 1	48,03576	16,97165
17	Pötzneusiedl 2	48,03872	16,97224
17A	Pötzneusiedl 2A	48,04026	16,97038
18	nach Hollern	48,06820	16,88046
19	Altarm vor Rohrau	48,06951	16,87223
20	Götzendorf 1	48,01476	16,59203
21	Götzendorf 2	48,01104	16,58458
22	Maria Ellend	48,11433	16,66258
23	Altarm 2	48,02917	16,82417

24	Schlossteich Rohrau	48,06389	16,85222
25	Orth an der Donau "Entenhaufen"	48,07257	16,421101
26	Teich Jetski 1	48,01751	16,495
27	Teich Jetski 2	48,01751	16,49501
28	Teich Rohrau am Damm	48,12090	16,22768
29	Rohrau	48,41020	16,52088
30	Bachgasse (Arbesthal und Göttlesbrunn)	48,33000	16,43
31	Sankt Martins Therme und Lodge	47,80859	16,91678

Fig. 4 shows the locations along or near the river Leitha. Two further spots beyond this region were included as sampling sites: One of them, located at the river Danube, was chosen in order to have a comparison with previous studies. The other one was at the 'St. Martins Therme' (Seewinkel 1, 7132 Frauenkirchen, Austria).

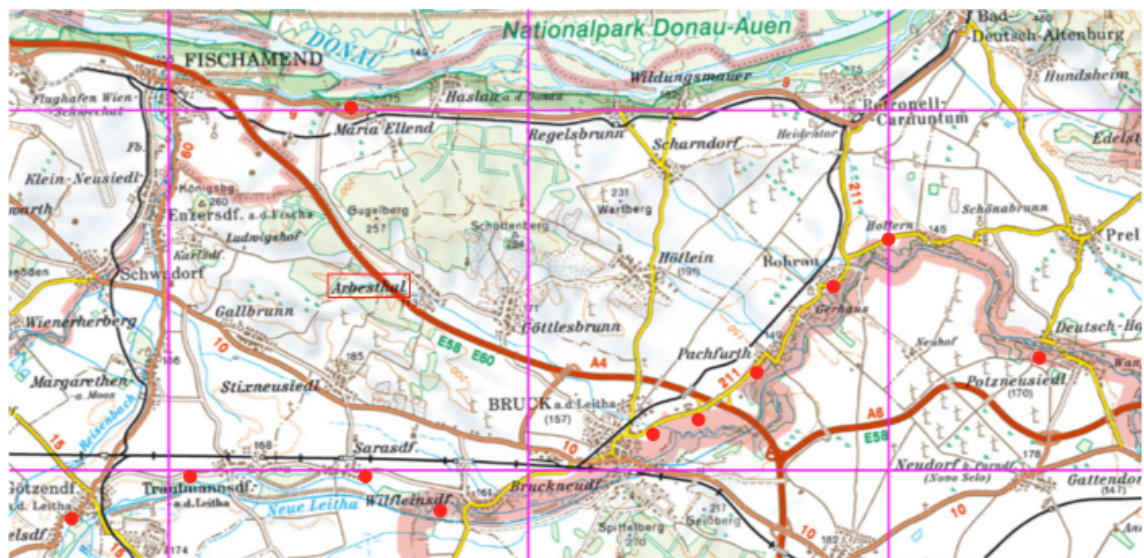


Fig. 4: Map of the sampled area along the river Leitha. The red points indicate the route along the river Leitha, from 'Götzensdorf' (in the west) to 'Potzneusiedl' (in the east). 'Arbesthal', the village near the planned wildlife crossing, is underlined. But not all sampling sites are marked.

Depending on the weather conditions, every two weeks, 5-10 locations were sampled by 1 to 4 people. The field trips usually took from 5 to 8 hours, spending 15 to 50 min on every single location. The different locations were characterized as plane riverbanks, puddles, flooded meadows and muddy soils. While collecting the snails, the time was measured with a stopwatch to get a rough estimate of the density of the snail

populations. The snails were picked up manually and were transported to the laboratory in glasses filled with water. In the laboratory the glasses with *G. truncatula* were stored at +8°C in the refrigerator, where the snails can survive up to one week. The glasses with *L. stagnalis*, *P. planorbis* and *P. corneus* were placed at room temperature, each individual in a single glass (250 ml) with water, near to a window for cercarial release experiments.

2.2. Parasitological examinations

The parasitological examinations of the collected snails were performed in a laboratory of the Natural History Museum Vienna. The shell heights of the different collected snail species were measured with a ruler to determine a possible correlation between parasite infestations and age/ size of the intermediate hosts.

Altogether, 339 individuals of *L. stagnalis*, 63 *P. planorbis* and 1 *P. corneus* were tested by cercarial release experiments. They were stored in separate glasses filled with water overnight in order to detect the release of schistosomatid cercariae. Infections in snails were determined by released cercariae in the water. All individuals of these species and 781 individuals of *G. truncatula* were dissected. For dissection, each snail was placed separately into a petri dish filled with water and then examined under a Wild/ Leica M3Z stereomicroscope (Leica Mikrosysteme, Austria). The respective snail was fixed through the use of forceps. In order to dissect the inner organs of the snail, the shell was broken and the soft body, especially the digestive organs, were cut into little pieces. The magnification varied between 60 fold (shell breaking) and 400 fold (examining the inwards). The parasitic stages of the affected snails were classified by light microscopy (Carl Zeiss, Austria) (Magnification 40 to 1000fold) and photography (Nikon). The infected parts of the snail (mostly the midgut gland) and/or released cercariae were immediately stored in 80% EtOH in an Eppendorf tube (1,5 ml– 2 ml) at -20°C. All samples were double checked microscopically by two persons. Depending on the degree of infection the dissection of one snail took between 2-20 min. The microscopic identification was made using the determination key of Mikes *et al.* (2001) (Fig. 5).

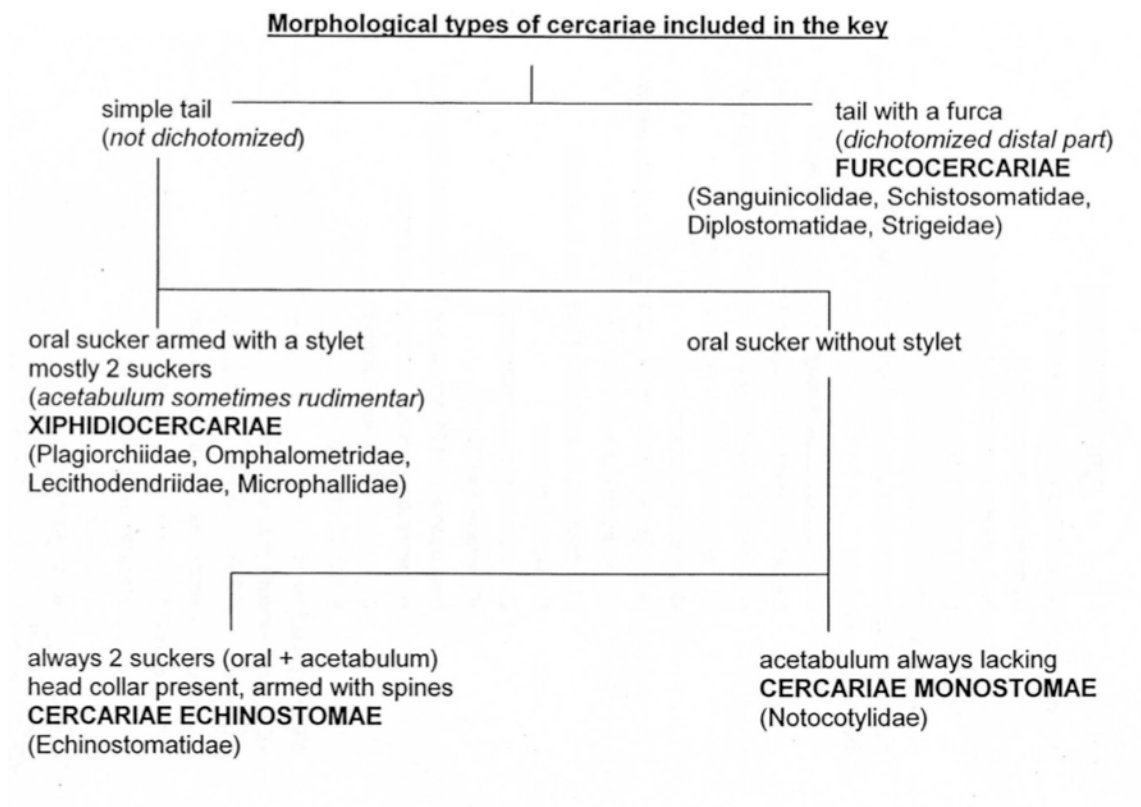


Fig. 5: Identification scheme for cercariae (Mikes *et al.*, 2001).

2.3. Molecular biological investigations

In order to verify the microscopic findings molecular techniques were used for general detection of trematodes and for species identification.

2.3.1. Primer design

Exact primers are essential for a successful PCR. In this work, two well-established PCRs from the literature were chosen. However, it was necessary to verify that the primers bind explicitly to the desired region without binding to the DNA of cestodes, turbellaria, bacteria or host species. For verification, a variety of different trematode sequences were downloaded from the GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>). In addition, to avoid cross-reactions, the primers were compared to other sequences from the NCBI database by blasting against nematodes, bacteria, cestodes, Digenea, Lymnaeidae and Anatidae (See Tab. 4).

Tab. 4: Selection of the used species and corresponding GenBank numbers.

Species	GenBank-No.
<i>Fascioloides magna</i>	EF051080
<i>Fasciolopsis buski</i>	L06668.1
<i>Calicophoron calicophorum</i>	L06566.1
<i>Fasciola hepatica</i>	X56041.1
<i>Paramphistomidae sp.</i>	FJ550131.1
<i>Lymnaea stagnalis</i>	AY427525.1
<i>Planorbis planorbis</i>	JQ776579.1

The chosen primer pairs were then ordered from Microsynth (<http://www.microsynth.ch/>).

For the detection of trematodes in general, the primer pair TremF/ TremR (TremF: 5'-GGT TCC TTA GAT CGT ACA TGC -3', TremR: 5'-GTA CTC ATT CGA TTA CGG AGC -3') was used, amplifying a fragment of the 18S rDNA, which is conserved in trematodes (Haider *et al.*, 2012). The expected size of the amplicon is approximately 430 bp. To ensure the specificity of the primers, the sequences were compared to other sequences from NCBI database by blasting against trematodes, hosts and bacteria. Tab.

5 summarizes the aligned sequences. Among these were *F. magna*, *F. hepatica*, *B. polonica* and *C. calicophorum*. *L. columella* was chosen as a representative of the family Lymnaeidae.

Tab. 5: Aligned sequences of the representatives of the superfamilies Echinostomatoidea, Paramphistomoidea, Schistosomatoidea and the family of the Lymnaeidae.

Superfamily	Species	GenBankNo.
Echinostomatoidea	<i>Fascioloides magna</i>	EF051080.1
Echinostomatoidea	<i>Fasciola hepatica</i>	AJ004969.1
Schistosomatoidea	<i>Bilharziella polonica</i>	AY157214.1
Paramphistomoidea	<i>Calicophoron calicophorum</i>	L06566.1
Family	Species	GenBankNo.
Lymnaeidae	<i>Lymnaea columella</i>	EU241866.1

The specificity test runs were carried out with already reference samples of *F. magna*, *F. hepatica*, Paramphistomum, *Taenia saginata* and *Taenia saginata* eggs. The sensitivity was tested with different dilutions of DNA. For this purpose the DNA was diluted with water in a ratio of 1:10, 1:100 and 1:1000 and it was executed with the ‘GastB52’ as well as with the ‘GastB56’ programme. The GastB PCR programmes are standard with 30 cycles, 1 min denaturation, 1 min annealing, 3 min extension. They differ in the annealing temperature of 52°C (GastB52) and 56°C (GastB56).

The second PCR TR98F/ TR98R (TR98F: 5'- CTC CGA CTG ATG ATG ACA AGA AGA-3', TR98R: 5'- ATG AGT GGC GAA CGG TAT CCT-3') was used for the detection of the genus *Trichobilharzia* (Korsunenko *et al.*, 2010). The primer pair amplifies the genome sequence ToSAU3A. The expected size of the amplicon is approximately 400 bp (Korsunenko *et al.*, 2010). These primers amplify only the DNA from cercariae and sporocysts of three trichobilharzian species. But the DNA of other digenean species (*B. polonica*) or the DNA of uninfected host snails (*L. stagnalis*) is not amplified (Korsunenko *et al.*, 2010). The primer pair was aligned with the three Trichobilharzian species *T. szidati* (GU980751), *T. franki* (GU980749) and *T. regenti* (GU980754). The test runs were carried out with a microscopically positive *Trichobilharzian* spp. sample (69: 17.7 ng/ µl). For this purpose, the DNA was diluted with water in a ratio of 1:10, 1:100 and 1:1000 and it was executed with the

‘GastB52’ as well as with the ‘GastB56’ programme. The specificity test runs were carried out with microscopically positive samples of *Trichobilharzia* spp. (69: 17.7 ng/ µl), *B. polonica* (67: 36.1 ng/ µl), *Echinostoma* sp. (68: 19.2 ng/ µl) and *L. stagnalis* (24.7 ng/ µl).

2.3.2. Samples

In total, 1,184 snails were collected. Out of them 781 *G. truncatula*, 66 *L. stagnalis*, 3 *P. planorbis* and 1 *P. corneus* were examined by microscopy. In 123 (66 *L. stagnalis*, 53 *G. truncatula*, 3 *P. planorbis*, 1 *P. corneus*) snails trematodes and/ or parasite infections were suspected. All samples were named numerically (Tab. 6)

Tab. 6: Overview of the PCR tested samples of *Lymnaea stagnalis* (LS), *Galba truncatula* (GT), *Planorbis planorbis* (PP) and *Planorbarius corneus* (PC) tested by PCR. The samples are listed according to the sample name (numbered consecutively), date of sampling, location and snail species.

Sample	Date	Location	Snail	25	28.8.12	27	LS
1	19.7.12	27	LS	26	28.8.12	27	LS
2	19.7.12	27	LS	27	28.8.12	27	LS
3	19.7.12	27	LS	28	28.8.12	27	LS
4	19.7.12	27	LS	29	19.7.12	26	PP
5	19.7.12	27	LS	30	13.8.12	27	LS
6	19.7.12	27	LS	31	13.8.12	27	LS
7	19.7.12	27	LS	32	13.8.12	27	LS
8	19.7.12	27	LS	33	13.8.12	27	LS
9	19.9.12	27	LS	34	13.8.12	27	LS
10	19.9.12	27	LS	35	13.8.12	27	LS
11	19.9.12	27	LS	36	13.8.12	27	LS
12	19.9.12	27	LS	37	13.8.12	27	LS
(Identical to no.11)				38	13.8.12	27	LS
13	27.7.12	27	LS	39	13.8.12	27	LS
14	27.7.12	27	LS	40	13.8.12	27	LS
15	27.7.12	27	LS	41	13.8.12	27	LS
16	28.8.12	27	LS	42	13.8.12	27	LS
17	28.8.12	27	LS	43	13.8.12	27	LS
18	28.8.12	27	LS	44	13.08.12	27	LS
19	28.8.12	27	LS	45	13.8.12	27	LS
20	28.8.12	27	LS	46	13.8.12	27	LS
21	28.8.12	27	LS	47	13.8.12	27	LS
22	28.8.12	27	LS	48	13.8.12	27	LS
23	28.8.12	27	LS	49	06.09.12	27	LS
24	28.8.12	27	LS				

50	06.9.12	27	LS
51	06.9.12	27	LS
52	06.9.12	27	LS
53	06.9.12	27	LS
54	06.9.12	27	LS
55	06.9.12	27	LS
56	06.9.12	27	LS
57	06.9.12	27	LS
58	06.9.12	27	LS
59	06.9.12	27	LS
60	06.9.12	27	LS
61	06.9.12	27	LS
62	06.9.12	27	LS
63	13.8.12	27	LS
64	02.7.12	26	LS
65	02.7.12	26	LS
66	02.7.12	26	LS
67	18.6.12	19	PC
68	18.6.12	19	PP
69	18.6.12	19	LS
70	08.6.12	27	LS
71	22.05.12	6	PP
72	23.04.12	3	GT
73	23.04.12	3	GT
74	23.04.12	3	GT
75	23.04.12	6	GT
76	23.04.12	6	GT
77	23.04.12	6	GT
78	23.04.12	6	GT
79	23.04.12	6	GT
80	23.04.12	6	GT
81	22.05.12	6	GT
82	08.05.12	25	GT
83	08.05.12	25	GT
84	08.05.12	25	GT
85	08.05.12	25	GT
86	08.05.12	16	GT
87	08.05.12	16	GT

88	08.05.12	25	GT
89	08.05.12	16	GT
90	08.05.12	16	GT
91	08.05.12	6	GT
92	08.05.12	6	GT
93	08.05.12	16	GT
94	08.05.12	16	GT
95	08.05.12	16	GT
96	08.05.12	16	GT
97	08.05.12	6	GT
98	08.06.12	25	GT
99	08.06.12	25	GT
100	08.06.12	25	GT
101	08.06.12	25	GT
102	08.06.12	25	GT
103	18.06.12	29	GT
104	18.06.12	29	GT
105	18.06.12	29	GT
106	18.06.12	29	GT
107	18.06.12	29	GT
108	02.07.12	29	GT
109	02.07.12	29	GT
110	02.07.12	29	GT
111	02.07.12	29	GT
112	02.07.12	29	GT
113	19.07.12	29	GT
114	19.07.12	29	GT
115	19.07.12	29	GT
116	19.07.12	29	GT
117	19.07.12	29	GT
118	05.09.12	29	GT
119	18.10.12	29	GT
120	18.10.12	29	GT
121	18.10.12	29	GT
122	22.05.12	6	GT
123	08.05.12	16	GT
124	22.05.12	3	GT

2.3.3. DNA extraction

For the preparation of *G. truncatula*, *L. stagnalis*, *P. planorbis* and *P. corneus* the QIAmp DNA Mini Kit (QUIAGEN, Vienna, Austria) was used. In brief, 10–20 mg of the midgut glands were cut into small pieces with a sterile razor blade and transferred into a 1.5 ml reaction tube. After adding 180 µl ATL Buffer and 20 µl of Proteinase K the vortexed sample was incubated at 56°C over night. The next day, when the tissue was completely lysed, 200 µl AL Buffer was added and the sample was incubated in a thermo-mixer at 70°C for 10 min. After adding 200 µl 100 % EtOH the whole content was loaded onto a spin column and was centrifuged for 1 min. at 8,000 rpm. After washing it with 500 µl AW1 Buffer, the sample was centrifuged for 1 min at 8,000 rpm. The flow-through was discarded and the sample was washed again with 500 µl AW2 Buffer. After centrifuging the column for 3 min, the flow-through was discarded again and the spin column was placed into a new collection tube. The tube was centrifuged again for 1 min. Subsequently, the spin column was placed in a 1.5 ml reaction tube, 200 µl AE Buffer was added onto the spin column's membrane and was then incubated at RT for 5 minutes. In order to eluate the DNA, the sample was centrifuged for 1 min at 8,000 rpm. The isolated DNA was stored at -20°C.

2.3.4. PCRs

All pipetting processes were performed on a sterile bench (Heraeus, Germany). All primer pairs were used in a dilution of 10 pmol/ µl. The PCR reactions were run in 0.2 ml soft PCR tubes. For every sample a master mix per sample was mixed and filled up with distilled water to the reaction volume of 50 µl. The master mix contained per sample 5 µl PCR buffer (Solis BIODYNE), 5 µl MgCl₂ (25 mM), 1µl dNTP-Mix (20 mM of each, 20 µl, Solis BIODYNE), 11 µl distilled water, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 0.25 µl polymerase (Hot Fire DNA Polymerase I, 5 U/ µl, Solis BIODYNE). The input of DNA volumes varied between 1 and 6 µl. The required reagents and also the master mix were vortexed before use. The isolated DNA was mixed by tipping. After finishing the reaction mix, the PCR tubes were vortexed and shortly centrifuged (10 s, 2.500 rpm). It was ensured that the tubes contained no air bubbles. The PCRs were performed in a thermal cycler (Eppendorf). All PCRs were run with a standard GastB PCR programme (30 cycles, 1 min denaturation, 1 min annealing, 3 min extension) using an annealing temperature of 56°C for the trematodes

specific PCR and of 54°C for the *Trichobilharzia* spp. PCR. For activating the Hot Start Polymerase it was necessary to start the PCR with an incubation of 15 minutes at 95°C.

For the evidence of trematodes, 53 samples of *G. truncatula*, 66 samples of *L. stagnalis*, 3 samples of *P. planorbis* and 1 sample of *P. corneus* were tested by the universal trematode PCR (Haider *et al.*, 2012). For this purpose every sample was tested in three different dilutions (1 µl, 3 µl, 6 µl). Furthermore, 70 samples (66 *L. stagnalis*, 3 *P. planorbis* and 1 *P. corneus*) were further tested by the *Trichobilharzia* spp. specific PCR (TR98R/ TR98F; Korsunen *et al.*, 2010), also in three different dilutions (1 µl, 3 µl, 6 µl).

2.3.5. Gel electrophoresis

In an agarose gel electrophoresis, DNA is separated in an electric field according to its size. To analyse the PCR results, the amplification products were loaded onto 2% agarose gels of different sizes. Therefore, 1 g or 2 g agarose (Sigma- Aldrich Inc., Saint Louis, MO, USA), respectively, were solved in 50 ml or 100 ml 1 x TAE buffer (0,04 M Tris-Acetate, 0,001 M EDTA). This mixture was boiled on a magnetic stirrer. After a short cooling period, 3 µl or 5 µl GelRed (GeneOn GmbH, Germany) were added and by swivelling slowly, the entire liquid gel was mixed. GelRed is an ultrasensitive, stable and intercalating nucleic acid stain that is structurally closely related to ethidium bromide. The warm solution was poured onto a gel tray and combs were added to form slots. A pipette tip was used to remove bubbles. After approximately 45 min the gel had polymerised, the combs were carefully removed and afterwards the samples were loaded. The PCR products were mixed with 10 x DNA loading buffer (containing Bromophenol blue) at a ratio of 1:10 (loading buffer : PCR product) up to a volume of 30 µl and were filled into the slots. The first slot was always loaded with 27 µl step marker (Sigma- Aldrich Inc., Saint Louis, MO, USA) which is required to validate the lengths of the amplicons, negative and positive controls were also loaded. The gel was placed in a horizontal electrophoresis system (BIO-RAD Wide Mini-Sub Cell GT) containing enough 1 x TAE buffer to cover the agarose gel. The gels ran at approximately 130 V and 300 mA for 30-45 minutes. When the loading buffer had reached the lower margin of the gel, the electrophoresis was stopped. Afterwards the gels were exposed to ultraviolet light, where the intercalated GelRed fluoresce with an orange colour that

strongly intensifies after binding to DNA (Biotium, http://www.biotium.com/product/product_info/newproduct/gelred_gelgreen.asp). For sequencing the positive bands were cut out with a scalpel.

2.3.6. Purification of agarose gel bands

The amplicons were purified from the gels with the Xact DNA Gel extraction Kit (genXpress, Austria) following the manufacturer's protocol. In brief, the gel bands were weighed and three times the volume of QG buffer was added. After that the mixture was incubated for 10 min at 50°C. The completely dissolved yellow gel mixture was vortexed and an aliquot volume of isopropanol was added. The solution was transferred onto a spin column in a 2 ml collection tube and centrifuged twice for 1 min at 13,000 rpm. Subsequently the flow-through was discarded and 500 µl QG buffer was added. The spin column was centrifuged for 1 min at 13,000 rpm. The mixture was incubated for 2-3 min after the addition of 750 µl PE buffer. After two centrifugal runs (1 min, 13,000 rpm) the spin column was transferred into a reaction tube and the DNA was eluted in 50 µl EB Buffer. The DNA was stored at -20 °C until use it for sequencing PCR.

2.4. Identification

For the species identification of the three most common European trichobilharzian species (*T. regenti*, *T. franki*, *T. szidati*) a PCR was chosen, that amplifies the region including ITS1, 5.8S rRNA and ITS2 sequences with the primers ITS5Trem (5'-GGA AGT AAA AGT CGT AAC AAG G-3'), which is complementary to the conserved region at the 3' end of the 18S rRNA gene, and ITS4Trem (5'-TCC TCC GCT TAT TGA TAT GC-3'), which is complementary to the conserved region at the 5' end of the 28S rRNA gene (Dvorák *et al.*, 2002). The sizes of the amplicons are approximately 1914 bp (*T. regenti*), 1330 bp (*T. szidati*), 1555 bp (*T. franki*), respectively (Dvorák *et al.*, 2002). The primer pair was blasted against sequences of the host snails (*L. stagnalis*, *P. planorbis*, *P. corneus*) as well as sequences of *Trichobilharzia*-related species (*B. polonica*, *Allobilharzia visceralis*). The test runs were carried out with microscopically positive samples of *Trichobilharzia* spp. (69: 17.7 ng/ µl), *B. polonica* (67: 36.1 ng/ µl), *Echinostoma* (68: 19.2 ng/ µl) and *L. stagnalis* (24.7 ng/ µl). A total of 9 samples (no.

18, 22, 34, 36, 53, 58, 63, 69, 70) were further tested by ITS5/ITS4 PCR for species identification.

After performing the ITS5/ITS4 PCR, 7 samples (no. 18, 22, 36, 53, 63, 69, 70) were further tested by sequencing. In addition, after performing the trematode unisversal PCR, 5 samples (no. 102, 121, 123, 124, 67) were further tested by sequencing. Each sample was sequenced using two different DNA volume dilutions, 1 µl and 3 µl, respectively. The purified DNA was added to 1 µl of the respective primer (10 pmol), 2 µl Big Dye (Applied Biosystems, Austria) and filled up with distilled water to a total reaction volume of 10 µl. Before the PCR was started it was ensured by centrifugation that there were no bubbles in the reaction (10 sec, 2,500 rpm). Subsequently, the sequencing programme started with an initial denaturation phase at 96°C for 30 sec and continued with 30 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and elongation at 60°C for 4 min. After the PCR had finished the samples were shortly centrifuged (10 sec, 2,500 rpm). The products were transferred in medium sized (0.6 ml) reaction tubes and mixed with 1 µl 3M NaAC and 3 µl 100% EtOH. Then an incubation on ice of 17 min, the samples were centrifuged for 30 min, 12,000 rpm, 4°C. The supernatant was discarded and the remaining pellet was washed in 70 µl 70% EtOH. After a repeated centrifugation of 10 min (12,000 rpm, 4°C) the supernatant was carefully discarded and the pellet was air-dried with lids open for 5 min. After the addition of 20 µl Hi-Di Formamid (Applied Biosystems, Austria) the samples were air dried again for 5 min. Following the drying process, the samples were incubated in a water bath (5 min, 95°C, 0 rpm), shortly centrifuged (10 sec, 2,500 rpm) and later sequenced. For sequencing, the lids of the samples were cut off and placed in the sequencing tray. The sequencing was performed in an automated ABI PRISM 310 Sequencer (PE Applied Biosystems, Langen, Germany).

2.4.1. Data analysis

Sequencing errors were corrected by aligning the results of repeated sequencing runs. In order to identify the samples, all sequences were blasted against sequences of digenetic trematodes available in GenBank. Clustal is a multiple sequence alignment computer programme. With a multiple sequence alignment it is possible to compare 3 or more sequences. The amplicons of the universal trematode PCR were aligned with a GenBank reference sequence of *F.magna* (EF534989.1). Only one sample (no. 67) was aligned with the GenBank reference sequence of *B. polonica* (AY157214.1) All trichobilharzian samples were aligned with the reference sequences of *T. szidati* (GU980751). In a sequence alignment the DNA-sequences are arranged to identify similar regions. In order to detect differences between very similar sequences pairwise alignments were performed. Local alignments were conducted with BLAST (Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/>) and EMBOSS (European Molecular Biology Open Software Suite, <http://www.ebi.ac.uk/Tools/emboss/>), for calculating the degree of sequence identity. In order to be able to identify single nucleotide polymorphisms (SNP) of the comparisons groups, the comparison groups and the primer pairs (TremF/ TremR; TR98F/ TR98R; ITS5/ ITS4) were aligned by means of Clustal X and GeneDoc (Nicholas and Nicholas, 1997).

3. Results

In total, 1,184 snails were collected and examined. The snails were collected during 16 field trips from March to November 2012, at 29 locations in the area of the river Leitha, between ‘Pötzneusiedl’ and ‘Götzendorf’ and two further locations at ‘Orth an der Donau’ and ‘St. Martins Therme&Lodge’. Altogether, snails were found at 11 of the chosen 31 locations and included the species *G. truncatula*, *L. stagnalis*, *P. planorbis* and *P. corneus*.

3.1. Location-characteristics and weather conditions

Habitats were chosen near the water line at swampy shallow shores of slow running water. The 31 locations included 27 sampling sites directly on river banks (Leitha/ Danube), 2 ponds, 1 flooded meadow and 1 artificial lake (‘St. Martins Lodge &Therme’). The highest densities were found at ‘Rohrau’ (Location 29) and in ‘Hollern’ (Location 6). Both are typical habitats of *G. truncatula* and are characterized by muddy surfaces (Fig. 6[3]). At ‘Rohrau’ (29) the snails were partially found in the mud directly. In ‘Hollern’ (6) (Fig. 6[4]), only a small area was sampled. The rest of the bank was difficult to reach because of thick vegetation. No findings were recorded at the artificial lake at ‘St. Martins Lodge &Therme’.

The highest densities of *L. stagnalis* were found at ‘Teich Jetski 2’ (27) (Fig. 6[1]). This is a small pond in the floodplains of the river Leitha. Also a second pond was sampled (‘Teich Jetski 1’, Location 26); Fig. 6[2]), but it dried out in late July 2012. Nevertheless still a lot of shells of *P. planorbis*, *P. corneus* and *L. stagnalis* were found here.



Fig. 6: Sampling sites of the locations:
 [1] 27: 'Teich Jetski 2', [2] 26: 'Teich Jetski 1', [3] 29: 'Rohrau', [4] 6: 'Hollern'.

The average precipitation of the months March to November is given in Fig. 7. The monthly rainfall in June and July reached 80 mm and 90 mm, respectively. The average precipitation for the whole month August was less than 30 mm, in September it was 60 mm. In Fig. 8 the average temperatures in 2012 are expressed in degree Celsius (°C). The temperature measurements were taken over by 'Wiesl's weatherstation Vienna', Penzing, 1140 Vienna. From March to August, the monthly average temperature increased above 20 °C, afterwards the temperature declined continuously.

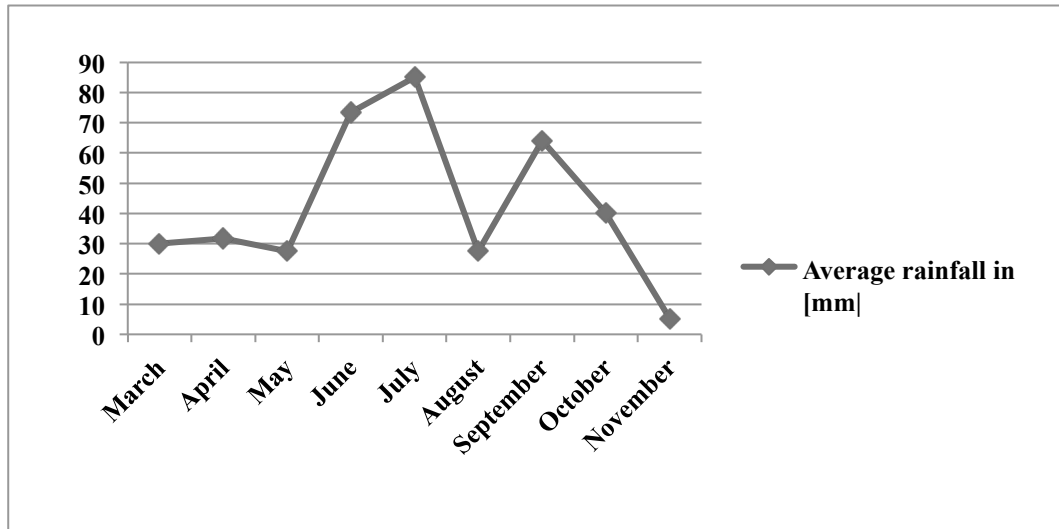


Fig. 7: Average precipitation from March to November 2012 in Vienna. The climatic data were measured by Wiesl's weatherstation Vienna, Penzing, 1140 Vienna.

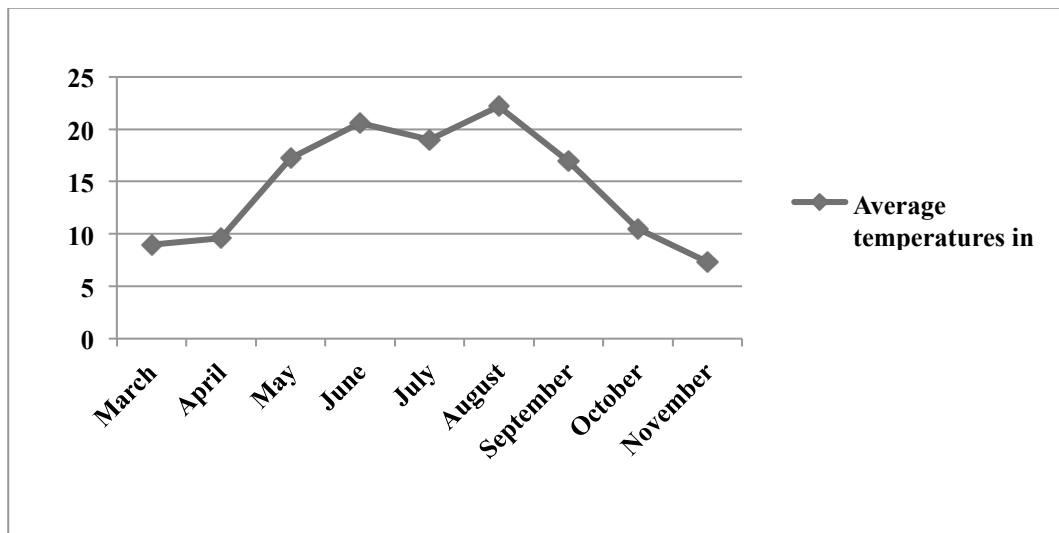


Fig. 8: Average temperatures from March to November 2012 in Vienna. The climatic data were measured by Wiesl's weatherstation Vienna, Penzing, 1140 Vienna.

3.2. Macroscopic results

3.2.1. Sampling

A total of 1,184 snails were collected. Thereof, 781 *G. truncatula*, 339 *L. stagnalis*, 63 *P. planorbis* and 1 *P. corneus* were found. The distribution of the collected snails per month is shown in Fig. 9. All *G. truncatula* (781 individuals) were found from April to October. The highest snail densities of *G. truncatula* were reached in June, afterwards it was only found occasionally. Furthermore, *L. stagnalis* (339 individuals), *P. planorbis* (63 individuals) and 1 *P. corneus* (1 individual) were collected from May to November 2012.

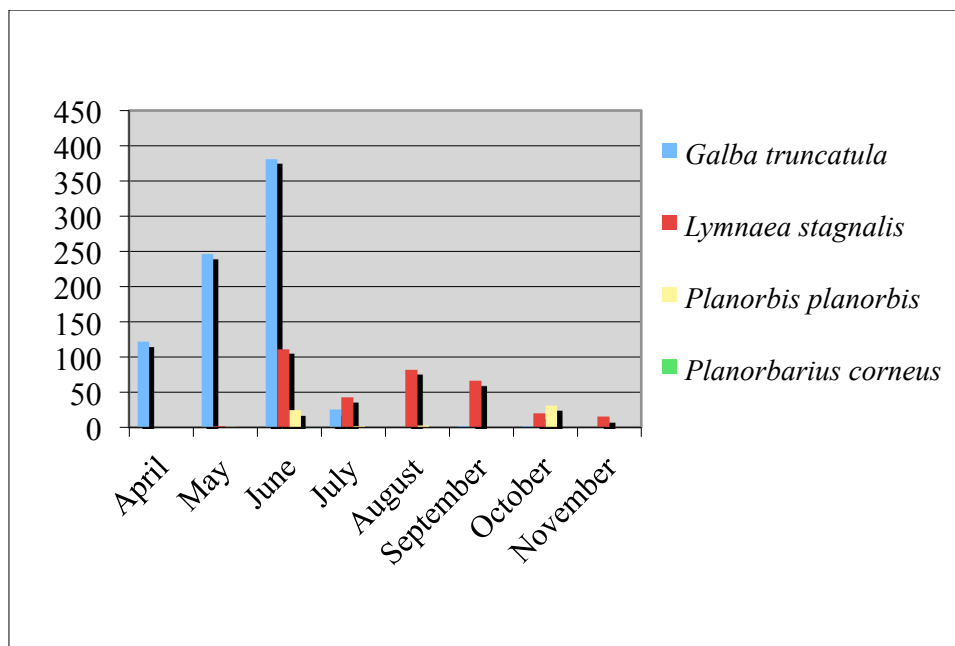


Fig. 9: Distribution of the collected freshwater snails from April to November 2012. The snail species are defined by following colours: Blue (*G. truncatula*), red (*L. stagnalis*), green (*P. planorbis*), purple (*P. corneus*).

Tab. 7 gives an overview of the local distribution of all sampled freshwater snails. It points out that the snail species looked for, were found only in 11 of 31 investigated locations. Most individuals of *G. truncatula* (322) were found at sampling site ‘Rohrau’ (29). Moreover, at location ‘Hollern’ (6) 171 individuals and at location ‘Potzneusiedl’ (16) 135 *G. truncatula* were collected. Less than 100 individuals were found at sampling sites ‘Leithazwickl’ (3), ‘Potzneusiedl 1’ (17), ‘Götzendorf’ (21) and ‘Entenhausen’ (25). Most individuals of *L. stagnalis* (270 samples) were collected at sampling site ‘Teich Jetski 2’ (27). The majority of the individuals of *P. planorbis* (41

samples) were also detected at this sampling site. The location ‘Altarm vor Rohrau’ (19) dried out in July, but 33 *L. stagnalis*, 4 *P. planorbis* and 1 *P. corneus* were found here in June.

Tab. 7: Overview of all collected snails (*G. truncatula*, *L. stagnalis*, *P. planorbis*, *P. corneus*) found at 11 locations.

	<i>G.truncatula</i>	<i>L.stagnalis</i>	<i>P.planorbis</i>	<i>P.corneus</i>
Leithazwickl (3)	58	-	-	-
Hollern (6)	172	-	1	-
Potzneusiedl (16)	135	-	-	-
Potzneusiedl 2 (17)	9	-	-	-
Altarm vor Rohrau (19)	-	33	4	1
Götzendorf (21)	1	-	-	-
Schloßteich Rohrau (24)	-	2	1	-
Entenhausen (25)	84	-	-	-
Teich Jetski 1(26)	-	34	16	-
Teich Jetski 2 (27)	-	270	41	-
Rohrau (29)	322	-	-	-
Total	781	339	63	1

Taking all findings together (Fig. 10), most snails were detected at the sampling sites ‘Rohrau’ (29) and ‘Teich Jetski 2’ (27). Less than 20% were found at the sampling sites ‘Hollern’ (6), ‘Potzneusiedl 1’ (16) and ‘Orth an der Donau’ (25). At samplings sites ‘Altarm vor Rohrau’ (19), ‘Schloßteich Rohrau’ (24) and ‘Teich Jetski 1’ (26) less than 60 individuals were found. These were summarized under the term “others”.

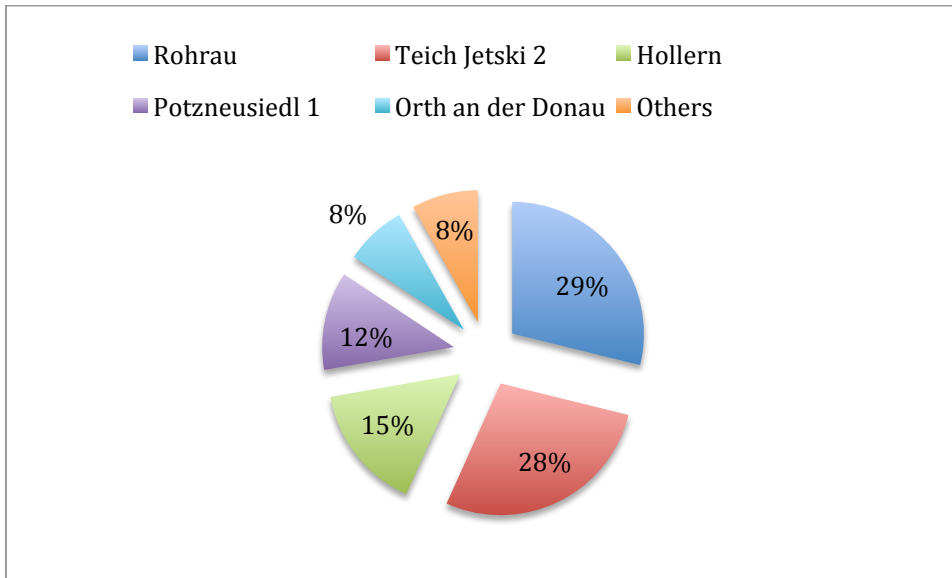


Fig. 10: Geographical distribution of all collected snails. The pieces are labelled with the number of the locations. The locations, where less than 60 snails were found, were summarized in „others“.

3.2.3. Density of the snail population on the basis of *G. truncatula*

The number of collected *G. truncatula* per 15 minutes per person (2-4) defines the density of the snail population. The snail density is counted as zero if no snails were found in this month. Fig. 11 gives an overview of the collected snails per person/ per month. *G. truncatula* was found in April, May, June, July, September and October 2012. The highest density of the *G. truncatula* was found in May and July. In these two months over 60 *G. truncatula* were collected within 10 min.

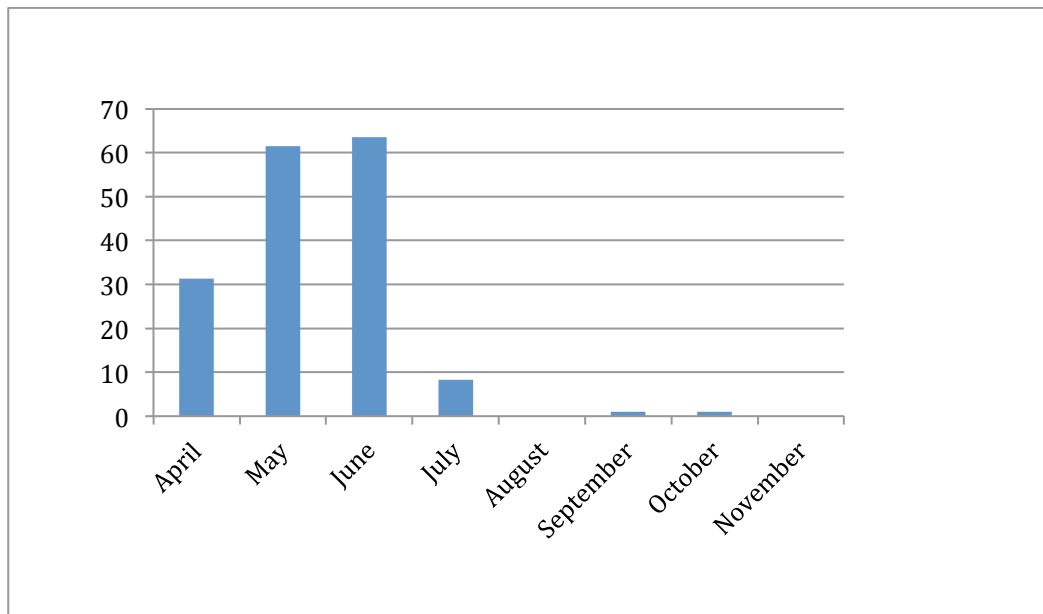


Fig. 11: Collected *G. truncatula* per person/ per month/ per 10 minutes from April to November 2012.

3.2.4. Shell size distribution of *G. truncatula*

Fig. 12 shows the distribution of the shell sizes of *G. truncatula* per month. The collected snails were classified in 4 size classes ($>1 - \leq 4$ mm, $>4 - \leq 6$ mm, $>6 - \leq 8$ mm, >8 mm). In general, in May most of the snails showed shell-sizes bigger than 4 mm. In July generally less snails were found, but it was recognized, that most of the collected snails showed shell sizes from 1 mm - ≤ 4 mm.

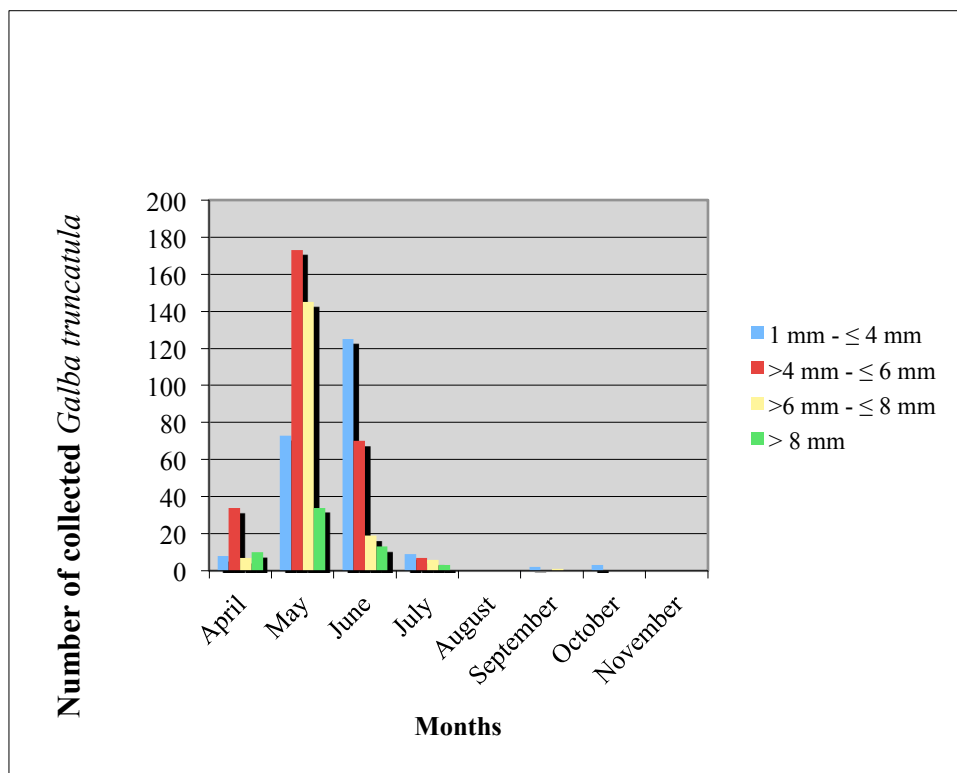


Fig. 12: Seasonal shell size distribution of the collected *G. truncatula*. The shell sizes were measured from the collected individuals from April to November 2012. The shell heights were classified in 4 size groups.

Fig. 13 summarizes the quantitative distribution of the shell sizes. The shell sizes were divided in 11 size classes. Most of the snails (167 individuals) were in the medium size classes (>4 mm to ≤ 5 mm). Only 25 individuals were smaller than 2 mm and only 17 individuals were ≤ 10 mm.

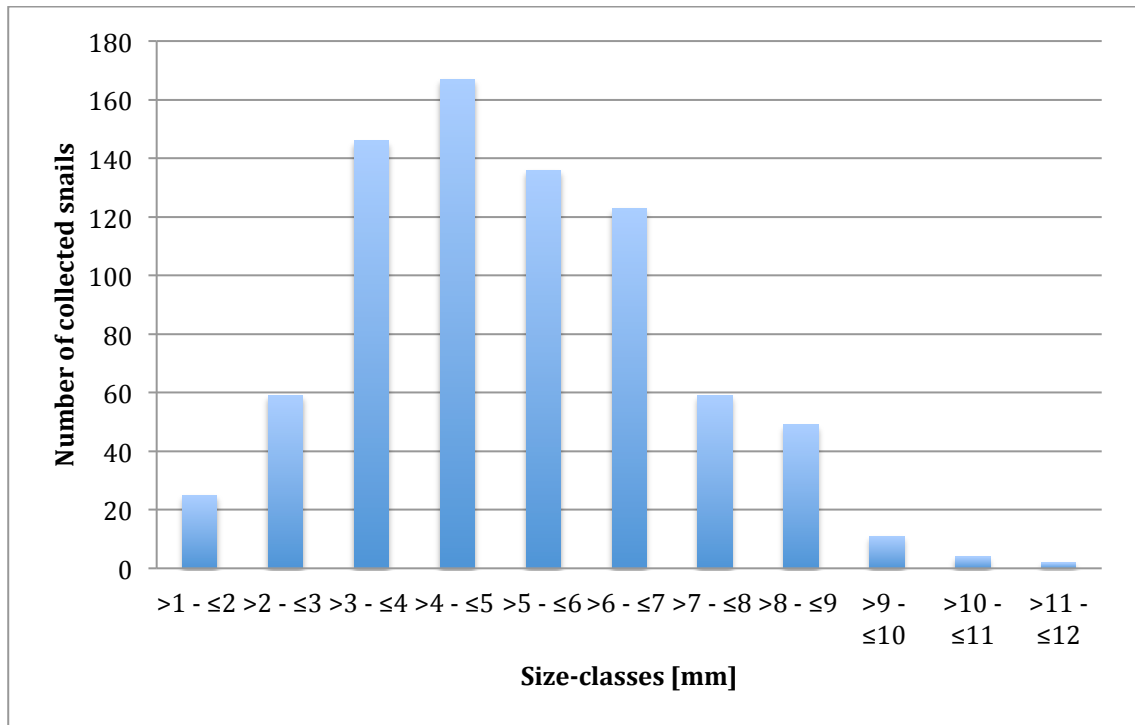


Fig. 13: Quantitative shell size distribution of the collected *G. truncatula*. The shell heights were classified in 11 size groups. The number is laid on the y-axis; the size classes are laid in the x-axis.

3.2.5. Shell size distribution on the basis of randomly collected *L. stagnalis*

In order to have a bigger sample size for shell size evaluation, also 27 empty shells of *L. stagnalis* were collected. Fig. 14 shows the distribution of the shell sizes of randomly collected *L. stagnalis*. In general, 10 shells showed shell sizes from > 2 cm to ≤ 3 cm, only one shell showed a shell size from > 5 cm to ≤ 6 cm.

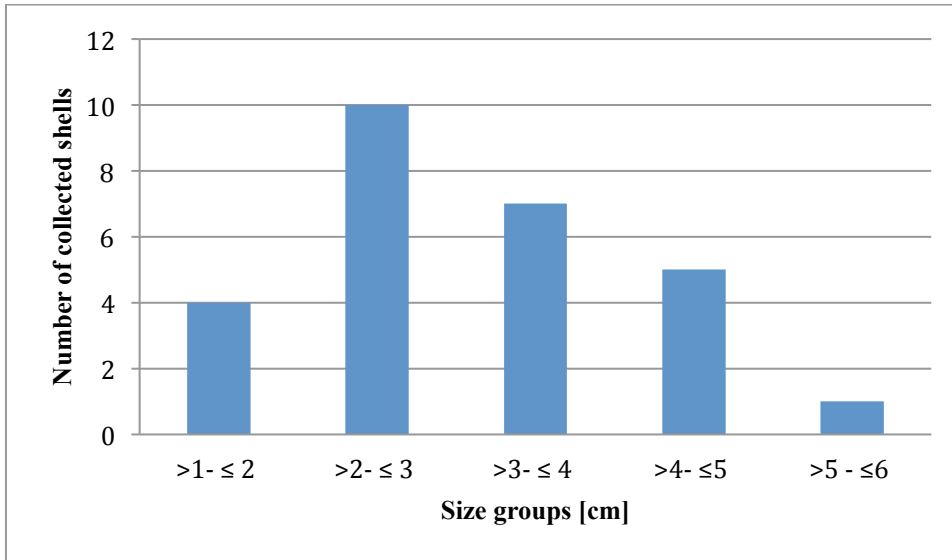


Fig. 14: Quantitative shell size distribution of randomly collected *L. stagnalis*. The shell heights were classified in 5 size groups. The number is laid on the y-axis; the size classes are laid in the x-axis.

3.3. Microscopy

Altogether, 70 snails were found to expel cercariae. These were 66 *L. stagnalis*, 3 *P. planorbis* and 1 *P. corneus*. Altogether, 66 *L. stagnalis*, 3 *P. planorbis*, 1 *P. corneus* and 781 *G. truncatula* were dissected. Out of these, 123 samples had a proven or suspected infection with digenean trematodes. These were 66 *L. stagnalis*, 53 *G. truncatula*, 3 *P. planorbis* and 1 *P. corneus*.

As far as identification was possible, 45 individuals of *L. stagnalis* were infected with Xiphidocercariae, 6 individuals with cercariae of Echinostomatidae (Fig. 15 [2,5]), 7 individuals with cercariae of Notocotylidae (Monostomae), 8 Furcocercariae. Out of the 8 Furcocercariae, 2 simultaneous infections of Xiphidocercariae and Furcocercariae (sample no: 11, 63 [Fig. 15 [4]]) and 6 *Trichobilharzia* sp. (sample numbers: 3, 18, 24, 64, 69, 70 [Fig. 3.10 [3]]), were suspected. Altogether, 2 of 3 individuals of *P. planorbis* were infected with Xiphidocercariae and 1 was infected with cercariae of Echinostomatidae. The single individual of *P. corneus* (no.67) was infected with cercariae of *B. polonica*.

Sporocysts were found in altogether 19 samples of *G. truncatula*. The other 20 trematodes, found in *G. truncatula* could not be reliably identified. Moreover, 2 samples infected with nematodes, 2 samples with Diptera larvae and 10 samples with the genus *Chaetogaster* (Fig. 15 [1]) were found.

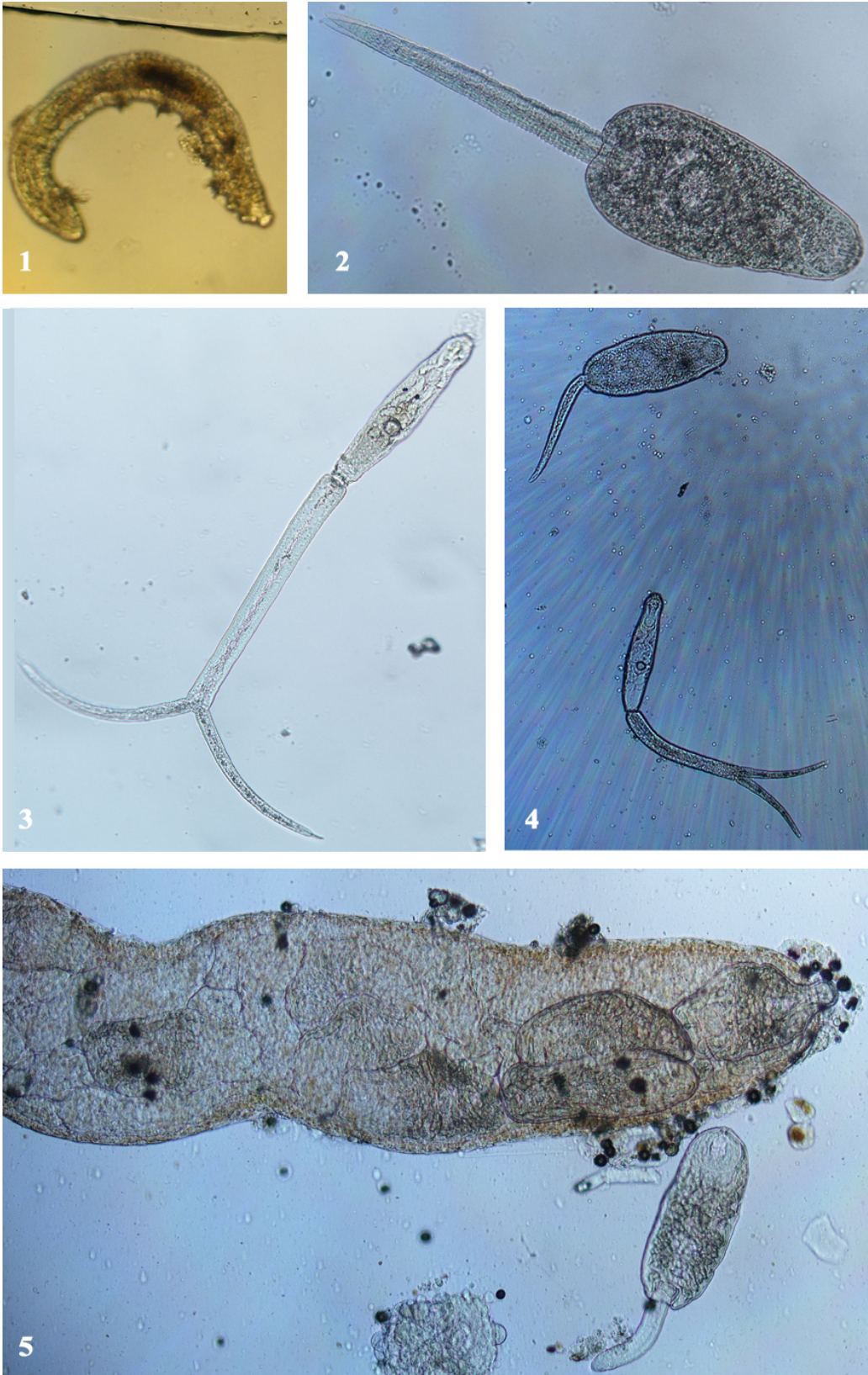


Fig. 15: Microscopic images of parasitic stages. [1] *Chaetogaster* sp., [2] Xiphidocercariae, [3] *Trichobilharzia* sp., [4] double infection with Xiphidocercariae and *Trichobilharzia* sp., [5] Sporocyst, with hatching Xiphidocercariae.

3.3.1. Distribution and seasonality of the infected snails

Fig. 16 shows a map of the sampled area. The area stretches along the river Leitha, from ‘Götzendorf an der Leitha’ (lower Austria) to ‘Pötzneusiedl’ (Burgenland). The location ‘St. Martins Therme & Lodge’ is not shown on this map. In total, snails with proven and/or suspected trematode infections were found at 8 sampling sites, marked with black circles.

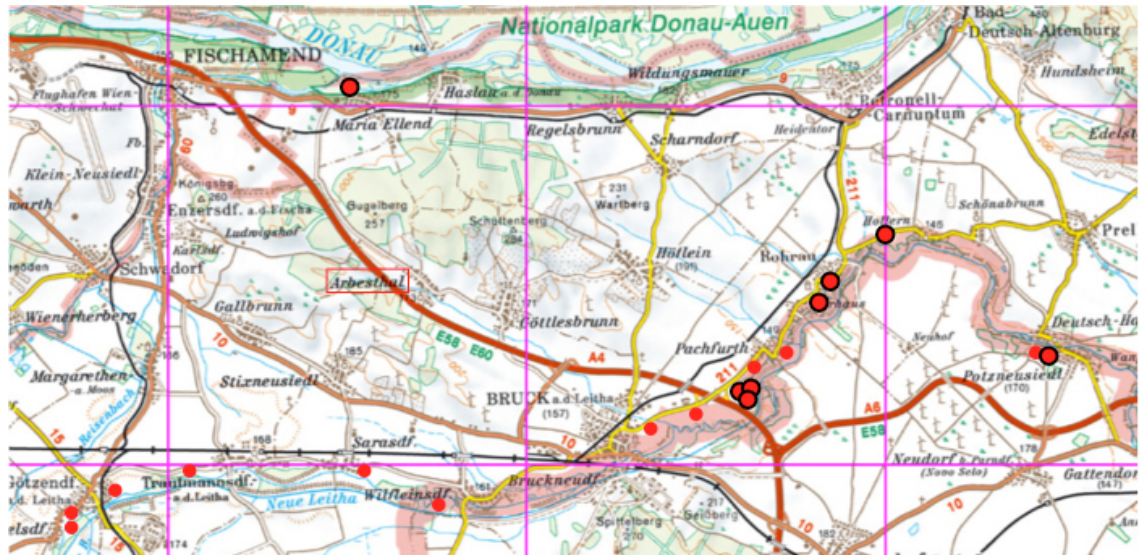


Fig. 16: Map of the sampled area. The sampling sites are marked with red points. All sampled locations were situated along the river Leitha. The sampling sites where infected snails were found are marked with black circles.

Tab. 8 shows the distribution of the infected snails at the 8 different sampling sites. A total of 61 infected snails were found at sampling site ‘Teich Jetski 2’ (27). Only 1 infected sample was found at sampling site ‘Leithazwickl’ (3).

Tab. 8: Infected snails were found at 8 of 31 sampling sites.

Location	Snails	Infected snails
Leithazwickl (3)	58	1
Hollern (6)	173	4
Potzneusiedl 1 (16)	135	5
Altarm vor Rohrau (19)	38	3
Orth an der Donau (25)	84	3
Teich Jetski 1 (26)	50	4
Teich Jestki 2 (27)	311	61
Rohrau (29)	322	13
Total	1.170	94

Infected *G. truncatula* were found from May until October 2012, with one exception in August. The 53 microscopically infected individuals of *G. truncatula* were distributed as shown in Fig. 17: Altogether, most infected snails (19/53 samples) were found in May, namely 5 individuals at sampling site ‘Orth an der Donau’ (25), 9 infected snails were detected at ‘Potzneusiedl 1’ (16), 4 infected individuals at sampling site ‘Hollern’ (6) and 1 infected individual at sampling site ‘Leithazwickl’ (3). Less than 3 infected snails were found in September and October at sampling site ‘Rohrau’ (29).

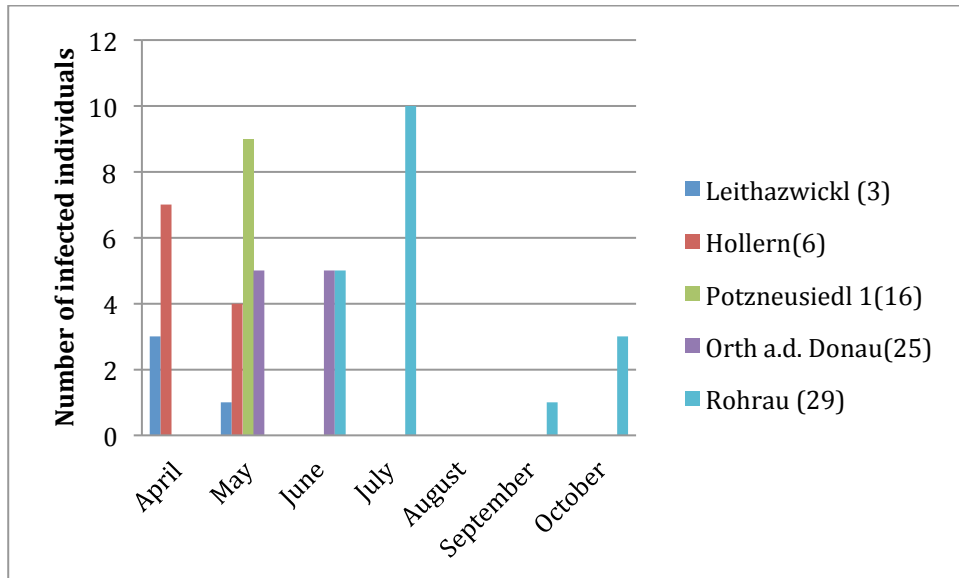


Fig. 17: The distribution of the 53 samples of *G. truncatula* that were microscopically infected.

L. stagnalis, infected with schistosomatid trematodes, were found from June until September 2012. In August 2012, 33 infected individuals of *L. stagnalis* were collected at location ‘Teich Jetski 2’ (27) (Fig. 18). However, infected *P. planorbis* were detected only in May and June 2012. At sampling sites ‘Hollern’ (6), ‘Altarm vor Rohrau’ (19) and ‘Teich Jetski 1’ (26), 1 infected *P. planorbis* was collected, respectively. In location ‘Altarm vor Rohrau’ (19) in June 2012, three different snails species (*L. stagnalis* (Fig. 18), *P. planorbis*, *P. corneus*) with three different cercariae (*Trichobilharzia* spp., Echinostomatidae, *B. polonica*) were found. In May, June and July 2012, 1 infected *P. planorbis* were found, respectively.

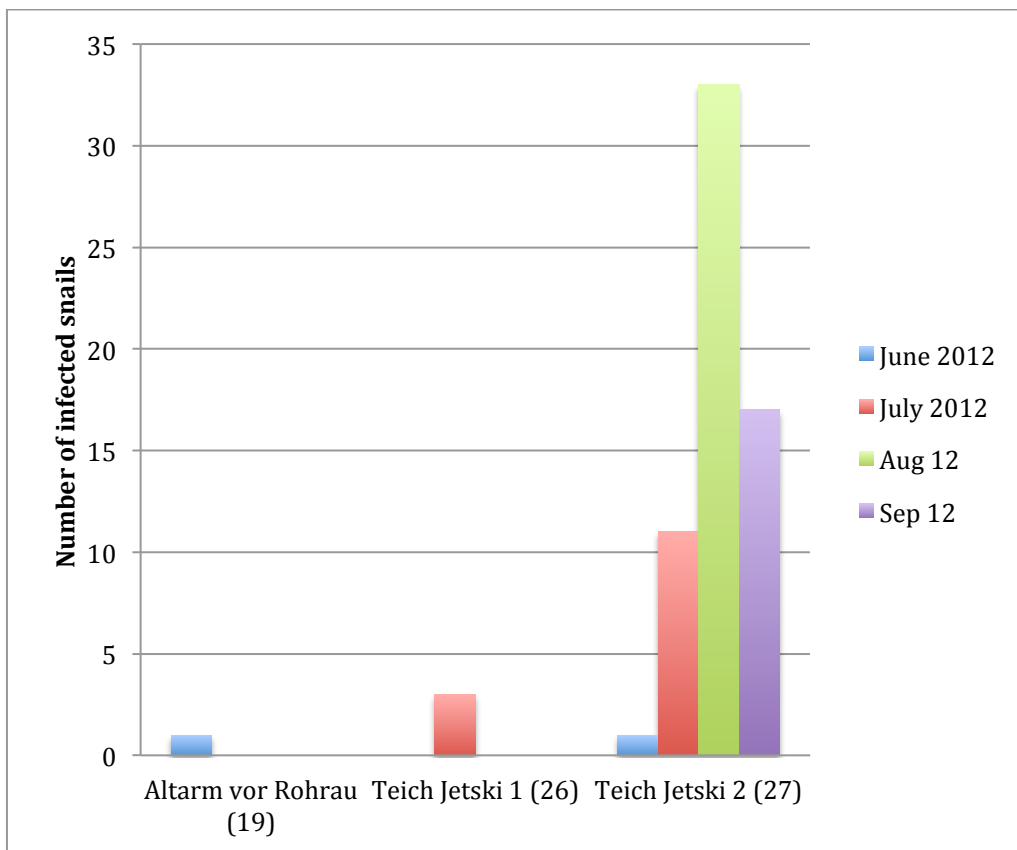


Fig. 18: In June 2012, only 1 infected *L. stagnalis* was found at sampling sites ‘Altarm vor Rohrau’ (19) and ‘Teich Jetski 2’ (27), respectively. In July, August and September 2012 more than 10 infected *L. stagnalis* have been found. In this graph, the number of infected snails is laid on the y-axis; the locations are laid on the x-axis.

In Fig. 19 is the seasonality of the infected freshwater snails shown. Most infected snails (33 individuals) were found in August 2012. However, in July infected *G. truncatula* (10 individuals), *L. stagnalis* (14 individuals) and *P. planorbis* (1 individual) were found. No infected snails were found in April and November 2012.

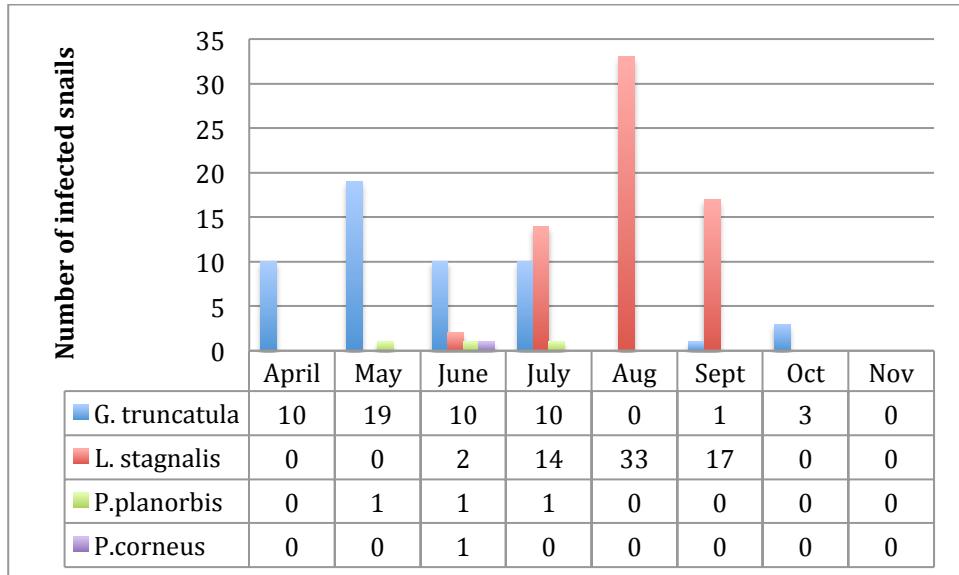


Fig. 19: The seasonality of the infected freshwater snails with trematodes, ordered according to months and numbers of the collected and infected snails. The number is laid on the y-axis; the months are laid on the x-axis.

3.4. PCRs

3.4.1. Sensitivity and specificity

The universal trematode PCR was tested for sensitivity using *F. hepatica* (292.02 ng/ μ l). This PCR detected as little as 0.2 ng of parasitic DNA. All three diluted DNA concentrations (1:10, 1:100 and 1:1000) gave clear bands of the correct size (Fig. 20).

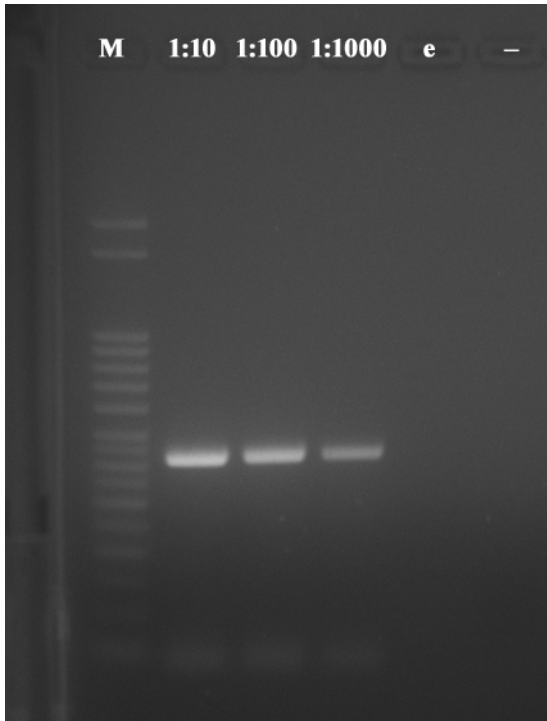


Fig. 20: Sensitivity test run of the primer pair TremF/ TremR in different dilutions (1:10, 1:100, 1:1000, e=empty, - = control).

Fig. 21 shows that the primers only react with *F. magna*, *F. hepatica* and other trematodes, but not with *Paraphistomum* or *T. saginata*.

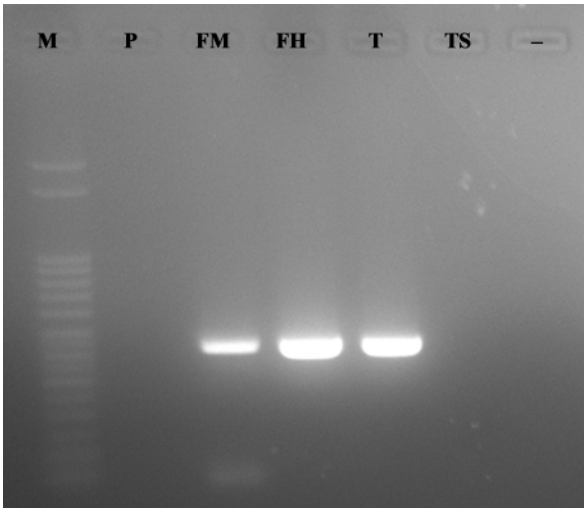


Fig. 21: Specificity run of the trematode specific primer pair TremF/ TremR. (M= Marker; P= Paramphistomum; FM= *F.magna*; FH= *F.hepatica*; T= unspecified trematodes, TS= *T. saginata*; - = control).

In Fig. 22 the alignment of the primer pair against *F. magna*, *T. franki*, *Paramphistomum* and *G. truncatula* is shown.

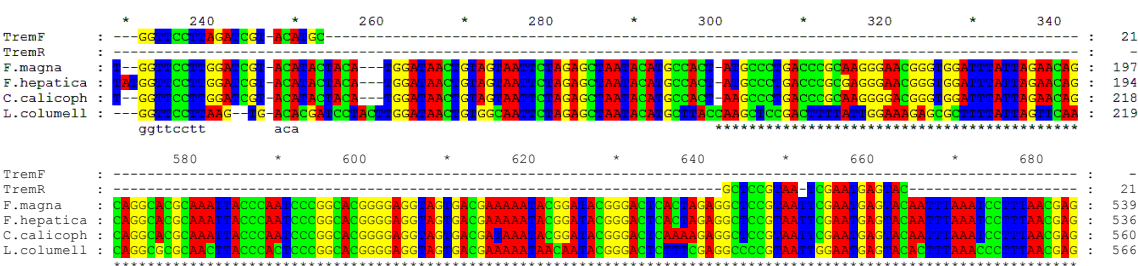


Fig. 22: Alignment of the trematode specific primerpair with reference sequences of *F. magna*, *T. franki*, *Paramphistomum* and *G. truncatula*.

Also the trichobilharzian specific PCR showed high sensitivity being able to detect DNA at all different dilutions of 1:10, 1:100 and 1:1000 (Fig. 23)

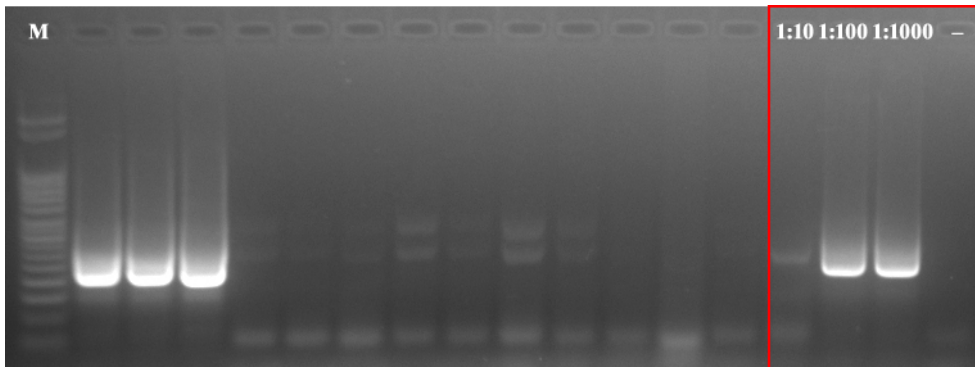


Fig. 23: The red framed amplicons show the sensitivity test run of the primer pair TR98F/ R in different dilutions (1:10, 1:100, 1:1000, - = control, M = Marker).

As shown in Fig. 24 the trichobilharzian specific PCR was indeed specific for *Trichobilharzia* spp. and did not react with *B. polonica*, *Echinostoma* and *L. stagnalis*.

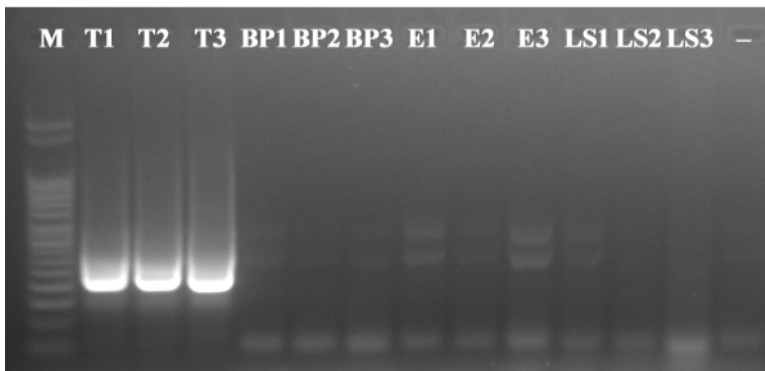


Fig. 24: Specificity test run of the primer pair TR98F/ R. (M=Marker, T1= *Trichobilharzia* sp. 1 µl, T2= *Trichobilharzia* sp. 3 µl, T3= *Trichobilharzia* sp. 6 µl, BP1= *B. polonica* 1 µl, BP2= *B. polonica* 3 µl, BP3= *B. polonica* 6 µl, E1= *Echinostoma* 1 µl, E2= *Echinostoma* 3 µl, E3= *Echinostoma* 6 µl, LS1= *L. stagnalis* 1 µl, LS2= *L. stagnalis* 3 µl, LS3= *L. stagnalis* 6 µl, -= Control).

3.5. Tested samples

All 123 snails with proven or suspected trematode infections were analysed by the universal trematode PCR (TremR/F). Of these, all 66 *L. stagnalis*, 3 *P. planorbis* and 1 *P. corneus* were positive. Only 13 out of 53 samples of *G. truncatula* were positive. The samples of the infected individuals of *G. truncatula* are listed by number, location and microscopic findings in Tab. 9.

Tab. 9: The samples of *G. truncatula*, that were tested positive by the trematode universal PCR. The samples are listed by location, date and microscopic findings.

Sample	Location, Date	Microscopic findings
89	Potzneusiedl, 08.05.12	Suspected sporocysts
90	Potzneusiedl, 08.05.12	Suspected sporocysts
91	Hollern, 08.05.12	Suspected sporocysts
92	Hollern, 08.05.12	Suspected sporocysts
93	Potzneusiedl, 08.05.12	Suspected sporocysts
94	Potzneusiedl, 08.05.12	Not reliable verified
102	Orth an der Donau, 08.06.12	Suspected sporocysts
105	Rohrau, 18.06.12	Suspected sporocysts
107	Rohrau, 18.06.12	Suspected sporocysts
116	Rohrau, 02.07.12	Suspected sporocysts
121	Rohrau, 18.10.12	Suspected sporocysts
123	Potzneusiedl, 08.05.12	Suspected sporocysts
124	Leithazwickl, 22.05.12	Suspected sporocysts

Fig. 26 gives an example of 4 samples (no. 121,123, 124 and 102) positive in the trematode PCR. Samples no. 121, 123, 124 and 102 showed a positive signal on the gel. Sample no. 124 showed only a positive signal with 3 μ l, but not with 1 μ l of DNA. Sample number 122 was negative, as already indicated by microscopy.

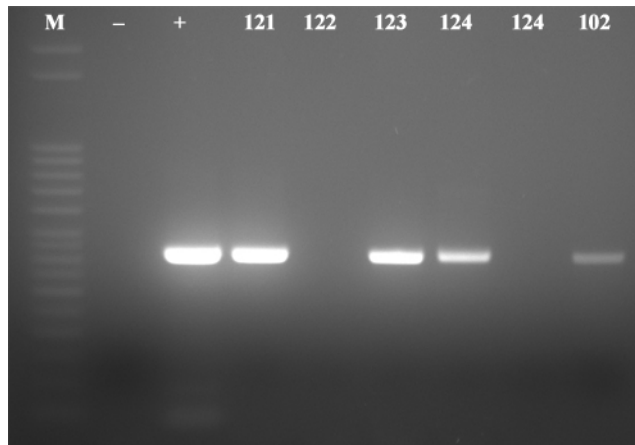


Fig. 26: Trematode PCR results after DNA extraction from microscopically positive (102,121,123,124) and negative (122) samples. Amplicons size: 430 bp.

Furthermore, all 70 cercariae found were analysed with a diagnostic PCR specific for *T. szidati*, *T. franki* and *T. regenti*. Samples no. 18, 22, 34, 36, 53, 58, 63, 69 and 70 had a band at approximately 390 bp (Fig. 27). Bands were strongest in samples no. 63 and 70. Sample 36 had a very bright band. Samples 18 and 69 are not shown on Fig. 27.

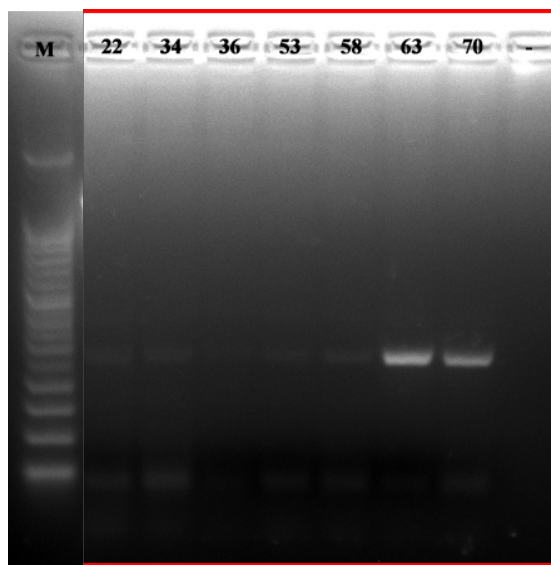


Fig. 27:Trichobilharzia specific PCR results from samples 22,34,36,53,58,63 and 70. Slots are labelled with sample numbers.

3.6. Identification

3.6.1. Identification by DNA sequencing

Altogether, 5 amplicons (no. 67, 102, 121, 123,124) of the TremR/F PCR were sequenced. The Alignment of sample no. 67 (suspected *B. polonica* in *P. corneus*) is not shown here. After the alignment with the reference sequence (AY157214.1), the classification *B. polonica* was possible.

The remaining 4 samples (all found in *G. truncatula*) originated from 4 different sampling sites ('Orth a. d. Donau' (no. 102), 'Rohrau' (no. 121), 'Pötzneusiedl' (no. 123), 'Leithazwickl' (no. 124). Altogether, 3 reliable sequences (no: 102, 123, 124) could be obtained. **Fig. 28** shows the alignment of the universal trematode PCR positive tested samples (102, 123, 124). This made the classification 'trematode' possible.

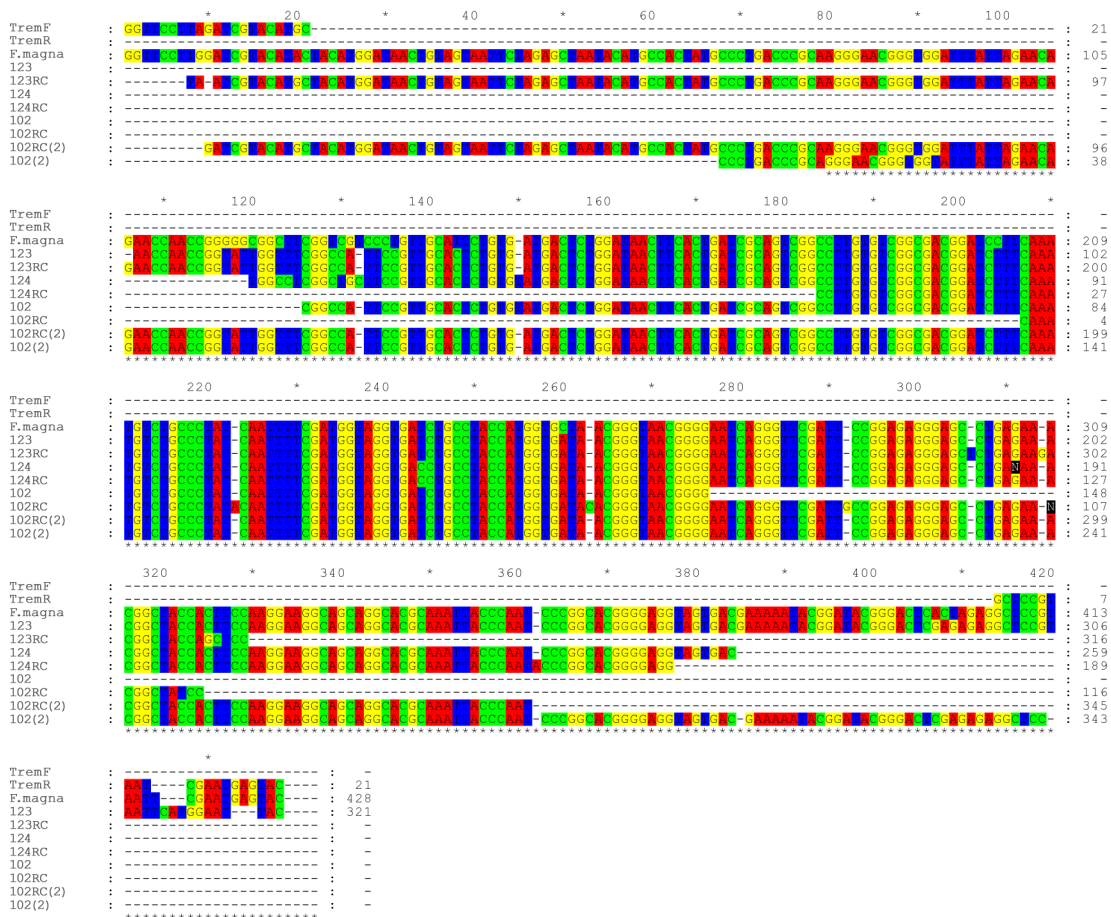


Fig. 28: Alignment of *G. truncatula* samples (102, 123, 124) with *F. magna* sequence from the database.

3.6.2. Identification by PCR

The 9 positive tested samples (no. 18, 22, 34, 36, 53, 58, 63, 69, 70) in the trichobilharzian specific PCR (TR98R/F) were analysed by a PCR (ITS5/4) in order to allow identification by sequencing the amplicon. As shown in Fig. 29 the PCR detected *Trichobilharzia* spp. and does not cross-react with *B. polonica*, *Echinostoma* and *L. stagnalis*.

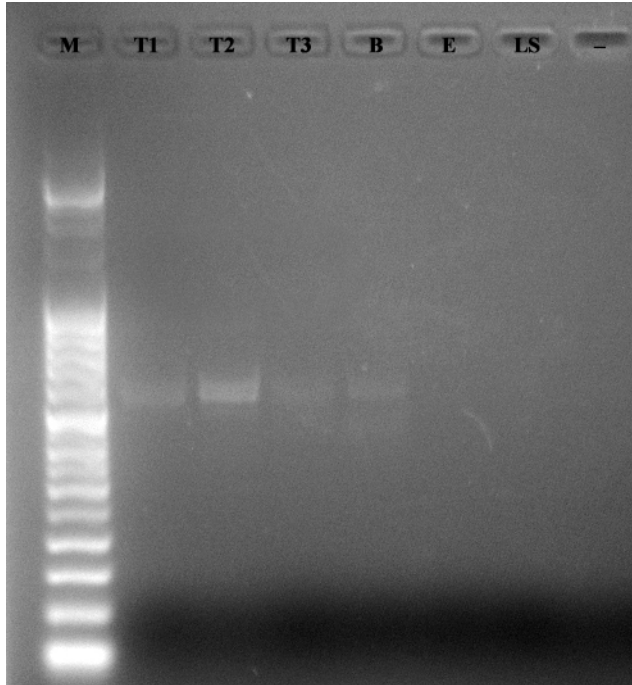


Fig. 29: Specificity test run of the primer pair ITS5/ 4 (M=Marker, T1= *Trichobilharzia* sp. 1 μ l, T2= *Trichobilharzia* sp. 3 μ l, T3= *Trichobilharzia* sp. 6 μ l, B= *B. polonica* 3 μ l, E= *Echinostoma* 3 μ l, LS1= *L. stagnalis* 3 μ l, -= Control).

Fig. 30 shows the results of the trichobilharzian PCR. The samples 18, 22, 36, 53, 63, 69, and 70 had a visible amplicon. Sample 34 was negative. Sample 67 (*B. polonica*) was used as a negative control and showed no amplicon. Sample 58 is not shown. This sample showed no amplicon, even after repeated PCR.

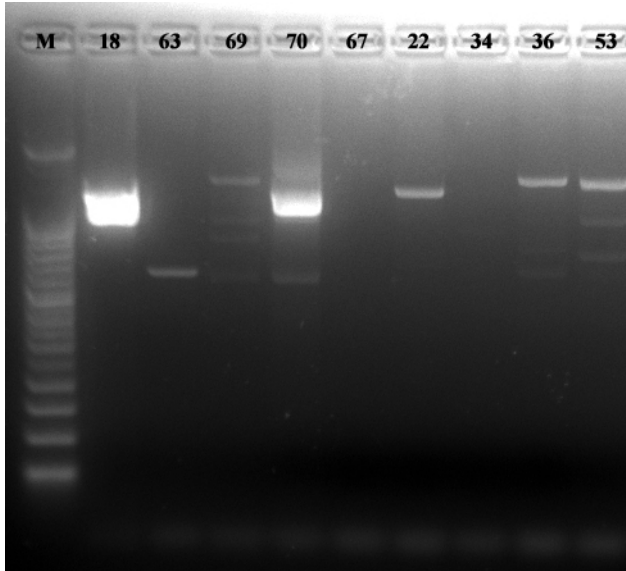


Fig. 30: PCR results after DNA extraction from microscopically trematodes positive samples. Slots are labelled with sample numbers.

Altogether, 7 samples (no. 18, 22, 36, 53, 63, 69 and 70) were positive in the ITS PCR. Of these 2 (no.18, 70) had a very strong band at approximately 750 bp. Whereby the amplicon of sample no. 69 was slightly shorter than the band of the other samples. Sample no. 63 had shorter amplicon at 600 bp. Sample no. 34 was negative. All amplicons were subjected to DNA sequencing and altogether 3 reliable (18, 69, 70) sequences could be obtained.

Tab. 10 summarizes the sequencing results of these three samples. Sequences were obtained from both strands in 3 independent set-ups. Altogether, 2 samples (no. 70, 69) were identified as '*T. szidati*', sample no. 18 could only be identified as '*Trichobilharzia* spp.' No reliable sequences could be obtained for samples no. 22, 36, 53 and 63.

Tab. 10: The sequencing results of the trichobilharzian specific positive tested samples. The samples are listed with location, date and the result of the sequencing.

Sample	Location/ Date	Seque[ncing
70	27, 08.06.12	<i>T. szidati</i>
69	19, 18.06.12	<i>T. szidati</i>
18	27, 28.08.12	<i>Trichobilharzia</i> spp.

3.7. Microscopy vs. molecularbiological techniques

Altogether, all collected *G. truncatula* (781 individuals) were examined by microscopy, of which 53 samples (prevalence 6.78%) had proven or suspected trematode infections. However, only 13 out of the 53 samples (prevalence 24.52%) were positive in the trematode universal PCR. Altogether, 4 samples from 4 different sampling sites were sequenced. All of these samples were positive for both techniques. Good sequences could be obtained for 3 samples (no. 102, 123, 124).

A total of 339 *L. stagnalis* were tested by cercarial release test, 66 individuals (prevalence 19.76%) expelled cercariae, all of them were examined by microscopy. All 66 samples were positive in the trematode universal PCR. 9 out of 66 samples were positive in the trichobilharzian specific PCR. The 9 samples were further tested by ITS5/ITS4. Out of them, 7 samples were positive. A total of 4 samples (no. 18, 63, 69, 70) were positive for both techniques.

Furthermore, 63 *P. planorbis* and 1 *P. corneus* were tested by the cercarial release test. Out of these, 3 *P. planorbis* (prevalence 4.76%) and 1 *P. corneus* expelled cercariae and were examined by microscopy. The 3 samples of *P. planorbis* were microscopically infected with Xiphidocercariae (no.29, 71) and cercariae of Echinostoma (no. 68). The 1 sample of *P. corneus* (no. 67) found to be infected with *B. polonica*, by microscopy. All of the 4 samples were positive in the trematode universal PCR and so positive for both techniques. Additionally, a good sequence could be obtained for samples no. 67 and the determination of the species '*B. polonica*' was possible.

3.8. Combined results of microscopy and molecular biology

3.8.1. Prevalence of digenean trematodes in freshwater snails

Altogether, 83 samples out of 1,184 freshwater snails were positive for trematodes. In total, Echinostomatidae were found in 6 samples of *L. stagnalis* and 1 sample of *P. planorbis* (Fig. 31). Monostomatidae were found in 7 samples of *L. stagnalis*. In 3 samples *Trichobilharzia* spp. were found. In 1 sample *B. polonica* was found. 47 snails were infected with Xiphidocercariae.

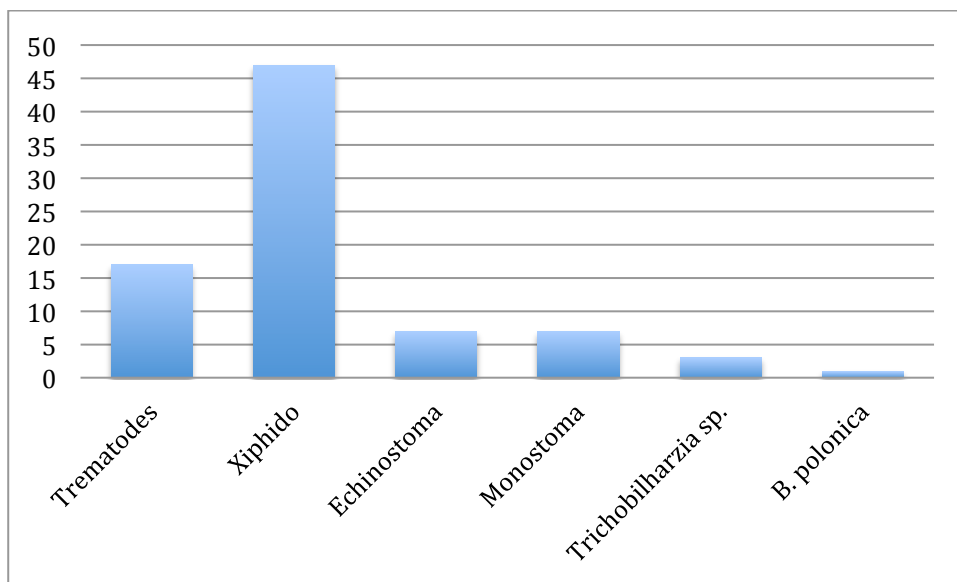


Fig. 31: Trematode families found in all samples, simultaneous infections refer to samples in which more than one type of cercariae were found. ‘Trematodes’ refer to unspecified trematodes.

3.8.2. Geographical and seasonal distribution of positive samples

The 82 positive samples were distributed over all investigated sampling sites (Fig. 32). The majority (78%) originated from the sampling site ‘Teich Jetski 2’ (27) where 61 samples were collected. Nevertheless, 5 positive samples (6%) were detected at location ‘Pötzneusiedl 1’ (16). At the location ‘Teich Jetski 1’ (26), only 4 positive samples were found (5%). Less than 3 positive samples were found at the sampling sites ‘Leithazwickl’ (3), ‘Hollern’ (6), ‘Altarm vor Rohrau’ (19), ‘Orth an der Donau’ (25) and ‘Rohrau’ (29).

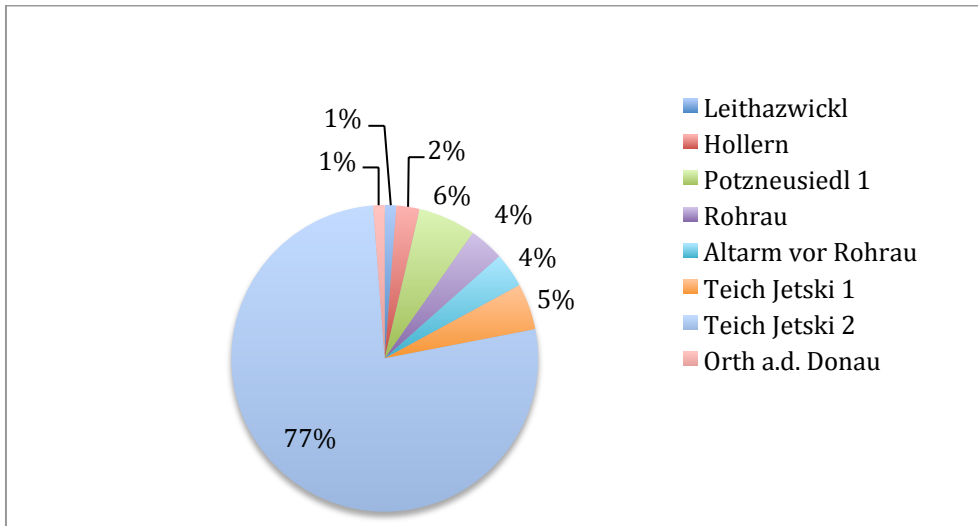


Fig. 32: Geographical distribution of infected snails.

Fig. 33 shows the seasonal distribution of the infected snails. In May, only 9 trematode positive samples were found. In June 4 positive samples were detected. Moreover, in July 15 infected snails were found. Most positive samples derived from August (33 positive samples). In September, an equal number of positive samples (17 samples) were collected.

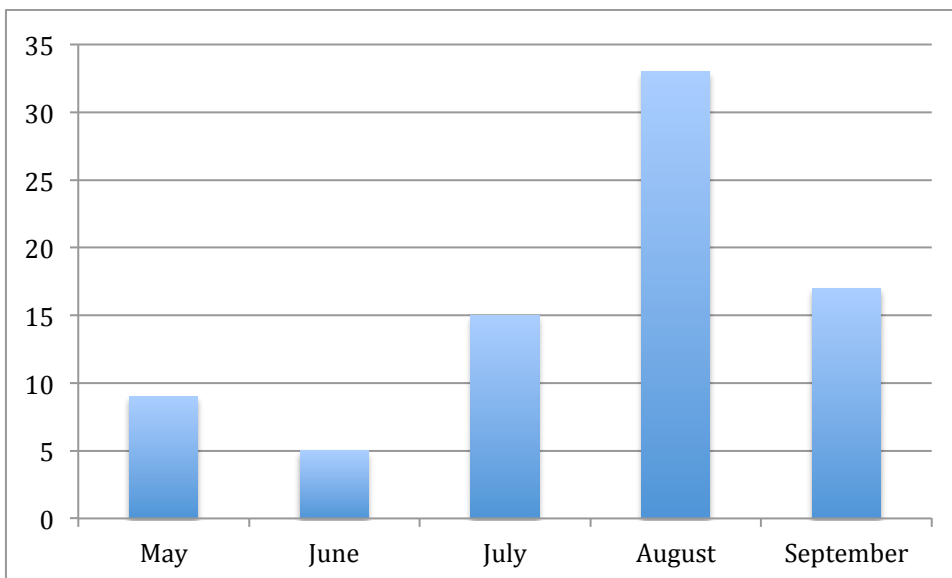


Fig. 33: Combined seasonal distribution of all dated positive samples. In the months May to September infected snails were found.

3.8.3. Prevalence of Schistosomatidae in freshwater snails

Altogether, 70 samples out of 403 collected freshwater snails, which can act as a possible intermediate host for Schistosomatidae, were positive for trematodes. As shown in Fig. 34, out of that, 3 of 67 *L. stagnalis* were positive for *Trichobilharzia* sp. (prevalence 5.97%) (1 *Trichobilharzia* sp., 2 *T. szidati*). In 1 out of 1 collected *P. corneus* sample *B. polonica* was found (prevalence 100%). The remaining 66 samples (63 samples of *L. stagnalis* and 3 samples of *P. planorbis*) could only be determined as ‘trematodes’.

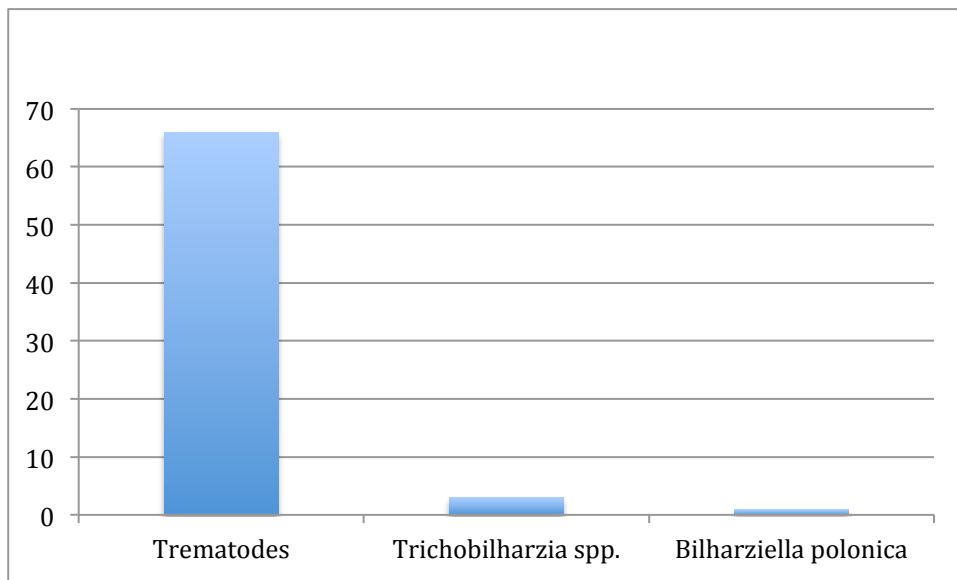


Fig. 34: Schistosomatids, that were found in the freshwater snails: ‘Trematodes’ refer to unspecified trematodes, *Trichobilharzia* spp. and *B. polonica*.

The geographical distribution of the infected Schistosomatidae was as follows. The 3 positive *L. stagnalis* samples of *Trichobilharzia* sp. and the 1 *P. corneus* sample of *B. polonica* were distributed over 2 investigated sampling sites (Fig.35). In total, 3 samples originated from the sampling site ‘Teich Jetski 2’ (27), and 2 samples were from sampling site ‘Altarm vor Rohrau’ (19).

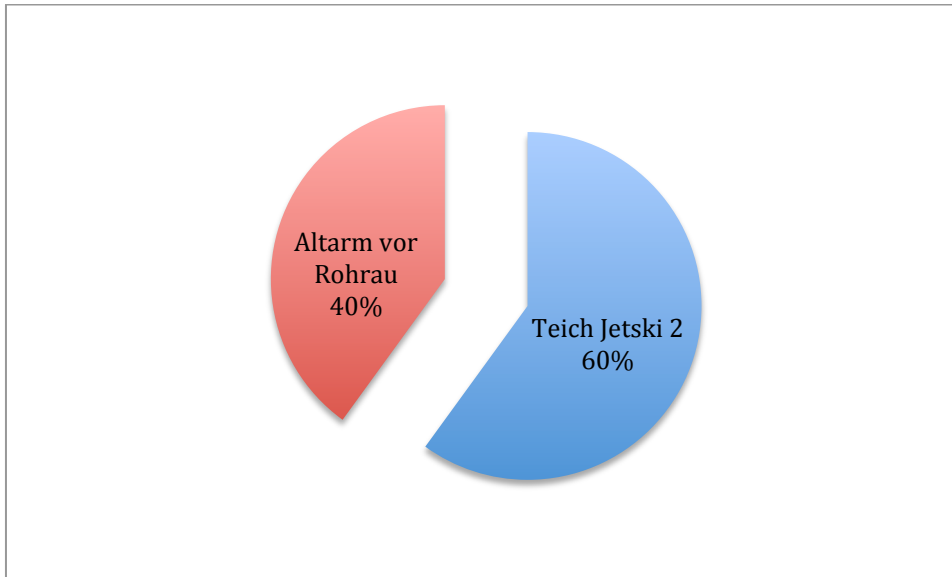


Fig. 35: Geographical distribution of with Schistosomatidae infected snails is distributed on 2 sampling sites. The majority of the infected lymnaeid snails (60%) were found at sampling site ‘Teich Jetski 2’ (27). The remaining 40% were collected at location ‘Altarm vor Rohrau’ (19).

The seasonal distribution of snails infected with *Trichobilharzia* spp. or *B. polonica* is shown in Fig. 36. Only in June and August positive samples were detected. The majority of the positive samples derived from June 2012. In August, only 2 infected snails were collected.

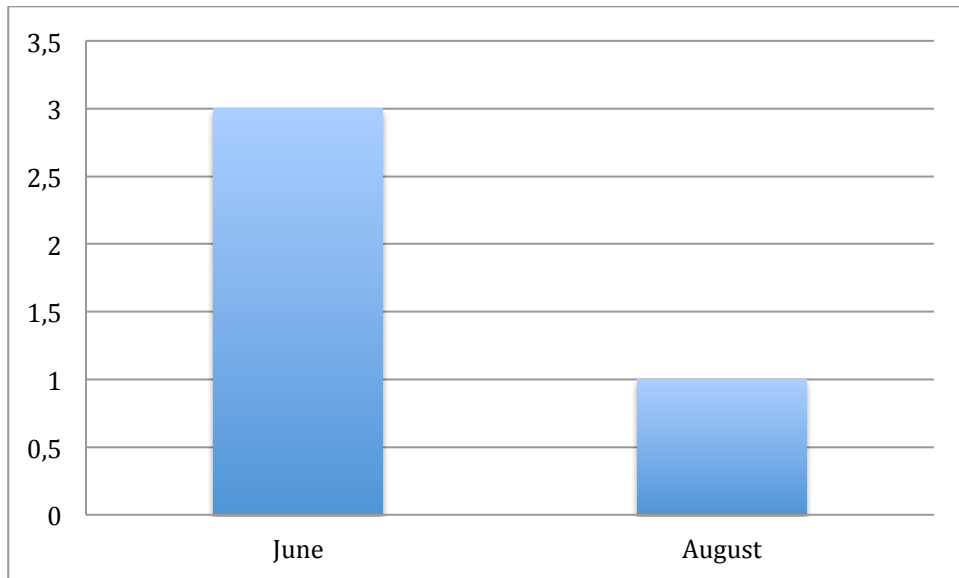


Fig. 36: Combined seasonal distribution of all dated snails infected with Schistosomatidae in the months June and August.

4. Discussion

In the current study, the occurrence of intermediate host snails and their infestation rates with trematodes in the floodplains of the River Leitha and its surrounding areas were investigated. The focus was given on introduced species such as *F. magna*, on native species such as *F. hepatica* and on the different trichobylarzia species.

4.1. Occurrence of *G. truncatula*, *L. stagnalis*, *P. planorbis* and *P. corneus*

In the current study in the floodplains of the river Leitha, the abundance of *G. truncatula* (781 individuals) was clearly lower than in a previous study in the immediately adjacent area of the Danube basin (Haider *et al.*, 2012, (3444 individuals)). Reckendorfer & Schäfer (2003) characterized the ideal habitat for intermediate host snails in the Danube backwaters as a muddy-sandy bottom, which is periodically flooded and closely located to flowing waters. These characteristics are also found in the floodplains of the river Leitha, for example sampling site ‘Rohrau’ (29), which is characterized by periodically flooded shores. At this sampling site, 41% (322/ 781 individuals) of all collected *G. truncatula* were found. However, the sampling site ‘Pötzneusiedl 1’ (16) was marked by muddy conditions and the proximity to the water. But at this sampling site, only in May 2012 snails (135/ 781 individuals) were found. It is reasonable that the collected snails had floated from upstream areas. Eckert *et al.* (2008) postulated, that passive transport is the most common distribution for snails. In contrast to the study of Konecny *et al.* (1999) (120 individuals) at ‘Schönau an der Donau’ (lower Austria), in the current study, *L. stagnalis* (339 individuals) was widely distributed in standing water bodies and small ponds such as at sampling sites ‘Altarm vor Rohrau’ (19), ‘Teich Jetski 1’ (26) and ‘Teich Jetski 2’ (27). These habitats were chosen because of the high population densities of *L. stagnalis*. All sampling sites were naturally, not artificially constructed ponds, located in the protected zone of the floodplains of the river Leitha. Additionally, these ponds are suitable habitats for nesting and stopover sites for migratory birds (Soldánová *et al.*, 2013). For these reasons, the ponds appear to be hot spots for high richness and infection levels of bird schistosomes (Soldánová *et al.*, 2013).

Around the same amount of individuals of *P. planorbis* was found in the current study (63 individuals) as in a previous study (51 individuals, Konecny *et al.*, 1999).

Significantly more individuals of *P. corneus* (73 individuals) were found in the study of Konecny *et al.*, (1999) than in the current study (1 individual). It is possible, that some snails were simply overlooked, but altogether the snail density seemed to be lower in the current sampling period. Additionally, the sampling site ‘Altarm vor Rohrau’ (19), where the species *L. stagnalis*, *P. planorbis* and *P. corneus* were found, desiccated early in summer 2012.

4.1.1. Reproduction cycle

Due to the relatively short sampling period, from April to November 2012, it is difficult to make a clear statement about the seasonal size distribution, particularly for *G. truncatula* which was only rarely found in the floodplains of the river Leitha. Here our results can only indicate trends. In May, more than 170 snails were found with a shell size from $>4\text{mm}$ to $\leq 6\text{mm}$. The biggest shell size measured 12 mm. Thus, it is likely, that these snails hibernated and belong to the generation of the previous year. In June, most of the snails had a height of about 2 mm, which suggests, that they had already reached sexually maturity and started reproduction (Smith, 1981). Also, the highest snail density of *G. truncatula* was found in June, which may correlate with a first reproduction peak. In previous studies in the floodplains of the river Danube, the shell size distribution implied a bimodal reproduction cycle with peaks in spring/ early summer and late summer/ autumn (comp. Haider *et al.*, 2012; Hörweg *et al.*, 2011). From June onwards, the size of the snail population declined. But possibly the snails were too small and were thus not found.

The highest densities of *L. stagnalis* and *P. planorbis* were found in June and in August, which may also correlate with the reproduction cycle. Of all collected *L. stagnalis*, 27 randomly chosen shells were measured to give a representative overview. Most of the snails had a shell size of $>2\text{cm}$ to $\leq 3\text{cm}$. This however indicates no significant evidence in relation to the reproduction cycle. The reproductive period of the snails lasts from the end of April until October (Heitkamp, 1982). One reason for the declined numbers in July might be the desiccation of the two locations ‘Altarm vor Rohrau’ (19) and ‘Teich Jetski 1’ (26).

4.2. Infection rates

In this study, a prevalence of 1.66% (13/ 781) of digenean trematodes in *G. truncatula* was found in the floodplains of the river Leitha. The findings include stages of *Paramphistomum* sp. and Echinostomatidae. Some trematodes remained undefined. In a previous study in the area of the floodplains of the river Danube, a prevalence of digenean trematodes of 2.41% in *G. truncatula* was found (Haider *et al.*, 2012). In the study of Haider *et al.* (2012), a prevalence of 1.83% of members of the superfamily Paramphistomoidea, a prevalence of 0.46% of the superfamily Echinostomatoidea, 0.09% of the order Strigeida and of 0.06% infected with Plagiorchiida was found. The considerably lower infection rates in the floodplains of the river Leitha may be a result of the rare occurrence of *G. truncatula* in this area. It is, however, also possible, that the snails were not found, although they occur in this area as much as in the surrounding areas. Of course, it is also possible that not all infections with trematodes were indeed detected. And also infection rates can vary between years.

In *L. stagnalis* a prevalence of 19.46% (66/ 339) was found. The prevalence included findings of Xiphidocercariae (13.27%), Notocotyliidae (2.06%) and Echinostomatidae (1.76%). The total prevalence of trematodes in *L. stagnalis* in our study (19.46%) is close to the average prevalence (23% in 296,180 host snails) of the combined data of 62 studies [(Kuris and Lafferty, 1994) cit. in Loy and Haas, 2001]. A considerably higher prevalence of digenean trematodes (44.9% in 43,441 *L. stagnalis*) was found in a pond system in southern Germany (Loy and Haas, 2001). The higher infection rates might result from the high abundance and the diversity of vertebrate and invertebrate hosts in that area (Loy and Haas, 2001). In *P. planorbis* a prevalence of 4.76% (3/63) was found. The most frequent findings were Xiphidocercariae (3.17%) and Echinostomatidae (1.58%). It is remarkable, that 1 out of only 1 collected *P. corneus* was infected by *B. polonica*. In a previous study, which was conducted in Central Europe, prevalences of 9.3% in *P. planorbis* and 35.6% in *P. corneus* were found. *P. corneus* was the most heavily infected snail species. They argue that this may reflect its body size, because *P. corneus* is the largest planorbid snail in Europe (Faltynková *et al.*, 2008). However, it must also be considered, that in the current study 2 habitats desiccated during the summer 2012. But, even after desiccation, numerous empty shells were found at these locations.

4.2.1. No occurrence of *F. magna* / *F. hepatica*

In the current study, no *F. magna*- / *F. hepatica*-infections were found in the floodplains of the river Leitha. Also in a previous study, *F. hepatica* was found only once (Hörweg *et al.*, 2011). In a previous study, a prevalence of *F. magna* of 17.7% in faecal samples of red deer in the floodplains of the river Danube was found (Liesinger, 2011). From 2001 until 2005, *F. magna* infections in deer have declined because a treatment programme was started. But in 2006, the prevalence increased again starting on the southern side of the Danube and in 2007 also on the northern side (Haider *et al.*, 2012). The reasons for this relapse are still unclear, but a formation of resistance in *F. magna* against triclabendazol is rather unlikely (Ursprung and Prosl, 2011). The results of our study indicate, that *F. magna* and *F. hepatica* do not (yet) occur in the investigated area. With the construction of the wildlife crossing and the resulting migration of the animals, the possibility is given that the parasites also spread into this area. After the completion of the wildlife crossing a further monitoring should be started to control the possible spreading (Sattmann *et al.*, 2014).

4.2.2. *Trichobilharzia* spp. / *Bilharziella polonica* in planorbid host snails

It is known, that the lymnaeids represent the most frequent and widely distributed intermediate hosts for the genus *Trichobilharzia*. In the current study, a prevalence of 0.88% (3/ 339) in *L. stagnalis* was found. In 1 out of 1 sampled *P. corneus* *B. polonica* (100%) was found. Also in the study of Loy and Haas (2001) the agents of cercarial dermatitis showed a constantly low prevalence (0.17% in *T. ocellata* in 43,441 examined *L. stagnalis*). They assumed, that such a low prevalence is normal in areas where cercarial dermatitis occurs in humans (Loy and Haas, 2001). Also in other previous studies, they found out, that in Europe, the prevalence of schistosomatids in snails is on low levels such as 0.05% to 5.0%. But the infection rates in birds were up to 74.5% [(Horák and Kolárová, 2011; Horák *et al.*, 2008) cit. in Soldanova *et al.*, 2013].

4.2.3. Other findings

In many samples of *G. truncatula*, *Chaetogaster* sp. was found. This oligochaete was always found below the shell or at the upper mantle rim of the snails. In total, 18 of 781 samples were infected with *Chaetogaster* sp. 8 samples of these were tested by the universal trematode PCR. None of the 8 samples were coinfecting with trematodes. This protecting effect had already been suggested in a previous study: an infection with *Chaetogaster* sp. protects the snail against invading miracidia (Fashuyi and Williams, 1977).

4.3. Seasonality of infections

Concerning fascioloid trematodes, in earlier studies, it was shown that *G. truncatula* acts as the intermediate host in the life cycle of *F. hepatica* and *F. magna*. The two-generation cycle of the lymnaeid snails indicates that two successive parasitic cycles could develop in the snails within one year. However, in the current study, altogether only few *G. truncatula* were found during the sampling season. Probably the number of snails was too low to record a significant seasonal distribution. During the investigation period, the highest monthly mean rainfall was in July, with over 80 mm per month. These are generally optimal conditions for *F. hepatica* infections of *G. truncatula* (Rapsch *et al.*, 2008). Additionally Rapsch *et al.* (2008), postulated that the optimal temperature range for infections is 22-25°C. In the current study (2012), these temperatures were reached only in August. Unfortunately, only a few *G. truncatula* were found in July at sampling site 'Rohrau' (29) and no *G. truncatula* were found in August.

Schistosomatid cercariae were found during the summer months, from June until September, in *L. stagnalis* and *P. corneus*. The highest infection rate was found in August. In this month 34 freshwater snails infected with digenean trematodes were found. August was the month with the lowest precipitation and the highest temperatures in the year 2012. It is known, that higher temperatures may enhance the cercarial production [(Kendall and McCullough, 1951) cit. in Mas-Coma *et al.*, 2009]). With increasing temperatures, the rate of physiological processes increases. The Q10 value (temperature coefficient) measures the rate of change of a biological system with increasing the temperature by 10°C [(Schmidt-Nielsen, 1997) cit. in Mas-Coma *et al.*, 2009]. A study on the influence of temperature on the cercarial emergence from their

snail host indicated that the Q10 values for temperatures around 20°C are higher than those for other physiological processes at the same temperature. This implies the cercarial output is not directly connected to the host metabolism. There have to be other factors that determine, how many cercariae are produced by time unit (Poulin, 2006).

4.4. Distribution of infections and infection risk for final hosts

In the current study, in the area of the river Leitha and in surrounding areas, many representatives of digenean trematodes were found. But, as mentioned above, no *F. magna* / *F. hepatica* infections were detected. In a GIS-based analysis (Reckendorfer & Groiss, 2006), four main risk factors for *F. magna* infections in deer were established: (I) wet pasturages, (II) periodical flooding to enable encystment of cercariae on vegetation, (III) high snail densities linked to dynamic waters and (IV) high densities of wild deer. In the current study, at sampling site 'Rohrau' (29) the risk factors were fulfilled: a muddy shore, where in total the most *G. truncatula* were found. Additionally, the prevalence of trematode infections was given at this location. But in fact, only a few hints of wild animals were found near the sampling site, so the actual infection risk for definitive hosts might be very low at this location. However, it should be noted, that the areas surrounding the river Leitha fulfil partly the conditions of the analysis of Reckendorfer and Groiss (2006). Thus, it is possible, that infected snails were already in this area, but they were not found at that time. Moreover, *F. magna* was already found with a prevalence of 0.23% (n=3,444) in the area Orth/ Danube (Haider *et al.* 2012). *F. hepatica* was found once in the study of Haider *et al.* (2012). Infections with *F. hepatica* in cattle and sheep are known mainly from the states Vorarlberg, Salzburg, Upper Austria, Styria and in the east of the Tirol (http://www.meduniwien.ac.at/hp/fileadmin/tropenmedizin/PDF_Christ/SteckbriefFasziolose.pdf). But human fasciolosis is very rare in Austria, only 1 or 2 cases are recorded per year. It is likely that the true incidence is higher (http://www.meduniwien.ac.at/hp/fileadmin/tropenmedizin/PDF_Christ/SteckbriefFasziolose.pdf). But taking into consideration that *F. hepatica* has large capacities to adapt new environments, as well as new intermediate and definitive hosts (Mas-Coma *et al.*, 2009) it is important to perform more monitoring in this area in the following years. Concerning schistosomatid trematodes, in previous studies as in the current study, the sampling sites for intermediate host snails were chosen on the one hand according to the

occurrence or suspicion of cercarial dermatitis in standing bodies of water. On the other hand sampling sites with high-populated intermediate host snails were chosen (Auer *et al.*, 1999). The artificially built lake of St. Martin's Therme & Lodge was investigated on account of suspected cases of dermatitis in this area. A bather asserted that he contracted cercarial dermatitis after bathing in this lake. After investigating the bank shores neither Lymnaeidae nor Planorbidae were found. Representatives of the Schistosomatidae use as intermediate hosts solely snails of these families (Auer *et al.*, 1999). At St. Martin's Therme & Lodge only the bivalve *Dreissena polymorpha* was detected. Therefore, the suspicion of the possible occurrence of cercariae of the family Schistosomatidae could not be confirmed. The highest prevalence of schistosomatid trematodes and the highest population density were found at sampling site 'Teich Jetski 2' (27). At this sampling site the avian final host as well as the intermediate snail hosts have been detected. These reasons would contribute to a higher infection risk for the definitive hosts. With a prevalence of 2.22% the actual infection risk for definitive hosts is rather high at this location. Adult schistosomes are detected mostly in waterfowl. Therefore it has been suggested that the human cercarial dermatitis is found most often along the major avian migratory flyways (Kolárová, 2007). In a study of Hörweg *et al.*, (2006) they created questionnaires to provide information about the causative agents, the waters, activities in the waters and details about the dermatitis itself. "The duration of stay in water and the kind of activity are not significant, but it can be assumed, that longer stays in shallow water increases the probability of infection (Hörweg *et al.*, 2006)." In the study of Hörweg *et al.* (2006), they recognized cases of cercarial dermatitis from May to August. In the current study, most infected snails with digenean trematodes, out of that 2 *Trichobilharzia szidati*, 1 *Trichobilharzia* sp. and 1 *B. polonica*, were found in June and August. But until now, it is not known, if there is a higher risk for the hosts to get infected by the genus *Trichobilharzia* or *Bilharziella*. In the study of Lichtenbergová and Horák (2012), they postulated, that there is a higher health risk for mammals for infections with *T. regenti*, the nasal schistosome, than with visceral schistosomes (*T. franki*, *T. szidati*). However, in the current study, only *T. szidati* was found. The one *Trichobilharzia* sp. could not be further determined. In Austria, at the moment there are only *T. szidati* (from *L. stagnalis*) and *B. polonica* (from *P. corneus*) proof records (Dvorák *et al.*, (1999).

For prevention it is often recommended to use sun lotions before bathing or to rub oneself dry after bathing (Hörweg *et al.*, 2006). But there is no evidence for these recommendations. Scientists of the University of Erlangen (Germany) produced a protective sun lotion. They examined, how the cercariae of *T. franki* and *T. szidati* recognize and penetrate the human skin. They found out, that two lotions showed positive effects. One of them, “Quallen + Sonnenschutz Canea” can be purchased in Germany. The other one, is a water-resistant sunscreen mixed with Niclosamide (<http://www.biologie.uni-erlangen.de/parasit/contents/research/Schutzcreme.pdf>).

Niclosamide is an anti-helminthicum, which uncouples oxidative phosphorylation and inhibits the oxygen uptake in invertebrates (Weinbach and Garbus, 1969).

5. Abbreviations

μl	-	microliter
A	-	adenine
BLAST	-	Basic Local Alignment Search Tool
Bp	-	base pairs
C	-	cytosine
CDC	-	Centers for Disease Control and Prevention
cit. in	-	cited in
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleotide
EtOH	-	Ethanol
G	-	Guanin
g	-	gram
g (force)	-	gravitational
ITS	-	internal transcribed spacer
M	-	mol
max	-	maximum
mg	-	milligram
min	-	minimum
min	-	minute(s)
ml	-	milliliter
mm	-	millimeter
NaAc	-	sodium acetate
NaCl	-	sodium chloride
NCBI	-	National Centre for Biotechnology Information
ng	-	nanogram
PCR	-	polymerase chain reaction
rDNA	-	ribosomal DNA
RNA	-	ribonucleic acid
Rpm	-	rotations per minute
RT	-	room temperature
Sp.	-	species
T	-	thymine

TAE	-	buffer consisting of Tris, acetic acid and EDTA
UV	-	ultra violet
V	-	volt

6. Glossary

- Adenine: A purine derivative. It is one of the major components of nucleotides and the nucleic acids DNA and RNA.
- Agarose: A polysaccharide gained from agar. It is used especially for gel electrophoresis.
- Alignment: Arrangement of DNA sequences.
- Alternation of generation: There are two or more different forms / generations within a life-cycle of an organism. These generations differ in habit, appearance and sexual/asexual reproduction.
- Amplicon: A short piece of DNA, that is a product of natural or artificial amplification.
- Amplification: A way in which genes can be overexpressed. Genetic amplification can happen naturally or artificially as in polymerase chain reaction, where short strands of DNA are amplified with the aid of enzymes.
- Cercariae: Tailed, free-swimming larval stage of trematodes. They are a product of asexual reproduction in a molluscan intermediate host.
- Chronic: A persistent and long-lasting disease or medical condition.
- Cladistic: A method of classification. Accordingly to their evolutionary relationships, animals and plants are placed into taxonomic groups called clades.
- Class: A category used in classification of organism. A class consists of similar or closely related orders.
- Classification: A method of taxonomy to group and categorize organisms into groups and determine their degree of relationship. A classification is hierarchical. The smallest group used is the species, which are grouped into genera. The hierarchy continuing up through tribes, families, orders, classes, and phyla to kingdoms.
- Cytosine: A pyrimidine derivative. Cytosine is one of the component bases of nucleotides and the nucleic acids DNA and RNA.
- Definitive host: The host in which the adult stage of a parasite develops.
- Digenea: Subclass of the Trematoda with alternation of generations and hosts.
- DNA (deoxyribonucleic acid): A nucleic acid consisting of two chains of nucleotides in which the sugar is deoxyribose and the four bases are adenine, thymine, guanine, cytosine. The two strands are wound round each other and

linked together by hydrogen bonds to form a spiral ladder-shaped molecule, which is called double helix. DNA is the genetic material, which is a major constituent of the chromosomes within the cell nucleus. It controls protein synthesis in cells and plays a major role in the determination of hereditary characteristics.

- DNA Hybridization: The process of joining two complementary single-strands of DNA to form a double-stranded molecule.
- Dorsal: A term to determine the anatomical structures. It's the upper side of a parasite.
- Ectoparasit: Organisms such as mites, lice, ticks, and other arthropods that live, at least temporarily, on the surface of the host's body.
- Electrophoresis: Movement of dispersed particles in an electric field. This technique is used for the analysis and separation of nucleic acids or proteins.
- Encystment: Development of a resistant external wall that helps the organism to survive dry periods or other environmental conditions.
- Endemic: Species that develop on biologically isolated areas such as islands because of their geographical isolation.
- Endoparasit: Parasite, who live within the host's body.
- Enzootic: Non-human equivalent of endemic. An organism is native to a habitat or a specific fauna.
- Enzyme: A protein, that catalyses biochemical reactions.
- Eosinophil: A polymorphonuclear leukocyte in which cytoplasmic granules stain red with use of Romanowsky- type stains
- Eosinophilia: Increasing number of eosinophils in the circulating blood. This results in an eosinophilic leucocytosis. This is frequently associated with tissue-invading helminths.
- Epidemiology: The study of the patterns, effects, causes of diseases.
- Epithelium: Cells that protect or enclose organs. Epithelium is one of the basic membranous tissues and is composed of single or multiple layers. It lines the cavities and surfaces throughout the body and forms many glands. Its tasks include for example secretion, protection, cellular transports.
- Eukaryote: An organism that consists of cells. Cells contain genetic material and a distinct nucleus.

- Excretion: Waste products of the metabolism and other non-useful materials, which are eliminated from an organism with the feces.
- Excystation: After excreting a cyst by a host, the parasite escapes from the cystic form for further development.
- Family: A taxonomic unit that contains genera and species.
- Fascioloidosis: A helminth disease caused by the trematode *F. magna*.
- Fasciolosis: Also known as Fascioliasis or Fasciolosis. Plant-borne trematode zoonoses, caused by two trematodes *F. hepatica* and *F. gigantica*.
- Faeces: Waste products of the metabolism and other non-useful materials, from an animal's digestive tract. Also known as excrement.
- Flame cell: A single cell with a tuft of cilia that extends into delicate tubule and represents part of the osmoregulatory system of trematodes. This cell is also known as a protonephridium and is involved with the excretion of excess water and other wastes. In living miracidia and adult worms, flame cells have a rapidly flickering movement.
- Flukes: see Trematoda
- Gelectrophoresis: A method to separate and analyse proteins by length to estimate the size of DNA fragments.
- GenBank: Sequence database with open access. In this database all until now known nucleotide sequences and their protein translations are accessible.
- Gene: A hereditary unit consisting of a sequence of DNA. The classical genetics defines a gene as a discrete particle, forming part of a chromosome. A gene determines a particular characteristic in an organism (e.g. tallness or shortness for the characteristic of height).
- Genome: The whole of genes of an organism's hereditary information. It includes the genes of DNA containing organelles and nuclear genes.
- Habitat: The place, where an organism or a population normally lives or occurs. Physical features or dominant plant types characterize a habitat.
- Helminth: Term, which includes nematodes (Nemathelminthes), trematodes and cestodes (Platyhelminthes).
- Hermaphroditism: In the same individual are the male and female reproductive systems present (Most flukes).

- Homolgy: Characters that are shared by a group of species because it is inherited from a common ancestor. In cladistics those characters are used to determine the evolutionary relationships of species or higher taxa.
- Host specificity: The degree to which a parasite may reach maturity in one or more host species.
- Infection: Invasion of any living organism by pathogenic microorganisms. The multiplication and establishment of the microorganisms can lead to tissue damage and disease.
- Infestation: A parasitic diseases caused by animals such as athropods or worms.
- Intermediate host: A host in the life cycle of some parasites in which required larval development occurs. Following this development, the larvae will be infective to the definitive host, or less frequently, a second intermediate host.
- Internal transcribed spacers-regions (ITS): A piece of non-functional RNA. The non-coding gene locus is situated between structural ribosomal RNAs on a common precursor transcript.
- Juvenile: Immature form of an individual organism that has not reached its adult form, sexual maturity or size.
- Larva: An immature stage that is clearly different morphologically from the adult. It requires further development and metamorphosis to reach the adult stage.
- Life cycle: Organisms of a particular species that undergo a period of events from the fusion of gametes in one generation to the same stage in the following generation.
- Marker: Gene or DNA-sequence with a known location on a chromosome. It is used to identify individuals or species.
- Metacercariae: A larval stage in most trematode life-cycles between the cercariae and adult worm. It encysts in tissues or on vegetation and is the infective stage for the definitive host.
- Miracidium: Ciliated embryo that develops in trematode eggs. This stage is infective for the snail intermediate host.
- Molecular systematics: Method to determine the evolutionary relationships of different organisms by using the data of amino acid sequences or nucleotide sequences.

- Mollusca: Large phylum of invertebrate animals. A soft and unsegmented body, differentiated into a head, a ventral muscular foot and a dorsal visceral hump, characterizes them. Molluscs occur in terrestrial, freshwater and marine habitats. The phylum consists of six classes including the Gastropoda, Bivalvia and Cephalopoda.
- Monogenea: Subclass of the Trematoda. The subclass is characterized by the absence of alternation of hosts or generations.
- Monophyly (monophyletic): A group of organisms consisting of an ancestral species and all its descendants. The group forms a clade and is the only type of group regarded as valid when constructing classification schemes.
- Morphology: Form and structure of organisms and their specific structural features.
- Nematoda: A phylum of pseudocoelomate invertebrates including the roundworms. An elongated, cylindrical body and an outer cuticle, which they shed four times during life to allow growth, characterize them. Within this phylum are free-living forms and disease-causing parasites such as *Filaria* (*Wuchereria*).
- Nucleic acid: Chain of nucleotides in living cells. There are two types of nucleic acid: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid).
- Nucleotide: Biological organic molecule that consists of a nitrogen-containing pyrimidine or purine base linked to sugar (ribose or deoxyribose) and phosphate group. Nucleotides form nucleic acids such as DNA and RNA.
- Operculum (operculated eggs): Specialized lid-like structure on parasite eggs through which the larval stages escape.
- Oral sucker: Muscular sucker at the anterior end of trematodes that surrounds the opening of the digestive tract.
- Paraphyly (paraphyletic): A group of organisms that excludes one or more descendants of a possibly single common ancestor.
- Parasite: Organism that lives in or on another and takes its nourishment from it.
- Paratenic host: This host is not required in the life-cycle of a parasite. But when its used by larval stages it enhances their opportunity for ultimately being ingested by a definitive host. Little or no necessary growth of larval stage occurs in this host.

- Pathogen: A microorganism such as viruses, many bacteria, fungi and protozoans, which can cause a disease.
- Phylogeny: The history and evolutionary development of a species or a group of related organisms.
- Phylum (pl. phyla): A category used in the classification of organism. In the taxonomic rank it is classified below kingdom and above class. Phyla consist of one or more several similar or closely related classes.
- Platyelminthes: A phylum of acoelomate invertebrate animals, comprising the flatworms. A flattened unsegmented body, a mouth that leads to a simple branched gut without an anus and a simple nervous system, characterizes them. The phylum includes many parasitic representatives such as the classes Turbellaria (planarians), Trematoda (flukes) and Cestoda (tapeworms).
- Polymerase chain reaction (PCR): technique in molecular biology for producing many copies of a DNA fragment.
- Polymerase: Enzyme, that catalyses the formation of polynucleotides of DNA or RNA. As a template the enzyme use an existing strand of DNA or RNA.
- Polyphyly (polyphyletic): A group, that is characterized by one or more character states which have converged or reverted so as to appear to be the same. But these characteristics have not been inherited from a common ancestor.
- Posterior: Term in anatomy, which describes the end of an organism opposite to its head.
- Prevalence: Term in epidemiology that defines the percentage of infected individuals in a population at a certain point of time.
- Primer: Short single-stranded DNA fragment. It is required as a starting point for DNA synthesis and binds to one end of a certain DNA sequence in a PCR system.
- Protonephridia: An excretory system of Platyhelminths for the elimination of liquid wastes. The system consists of flame cells and tubules.
- Protozoa: A group of unicellular or acellular eukaryotic organisms. Some of the organisms are parasites such as the agents causing malaria (plasmodium) and the sleeping sickness (Trypanosoma).
- Purine: A pyrimidine ring fused to an imidazole ring. Adenine and thymine belongs to the group of the purines.

- Redia (pl. rediae): A larval stage in the development of a trematode. Rediae already have a mouth, gut, central nervous system, salivary gland and a birth pore.
- Ribosomal DNA: Sort of DNA molecule in the ribosomes. It participates in protein synthesis.
- RNA (ribonucleic acid): A long, single-stranded chain consists of phosphate, ribose and the four bases adenine, guanine, cytosine and uracile. RNA molecules play a important role in protein synthesis. All living cells and many viruses possess RNA molecules.
- Sequencing (DNA sequencing, gene sequencing): A process to determine the nucleotide order of a DNA fragment. The Maxam-Gilbert method and the Sanger or dideoxy method are used techniques.
- Species: The basic unit of classification that defines a group of organisms. The biological species concept defines a species as a group of individuals that can usually breed among themselves and produce fertile offspring. It is possible that within a species, groups of individuals become reproductively isolated because of geographical or behavioural factors. These individuals may form a new and distinct species.
- Spectrophotometry: The quantitative measurement of the concentrations of DNA, RNA or proteins by light emittance.
- Sporocyste: a larval stage in many trematode worms.
- Stoma (pl. stomata): An epidermal pore in plant leaves, which is present in large numbers. The pores control the passage of gases into and out of a plant. Due to changes in the water content, the movement of semi-circular guard cells, by which each stoma is bordered, controls the size of the aperture.
- Swimmer' itch: Also known as cercarial dermatitis. Papular dermatitis in humans caused by the cercariae of bird schistosomes. They penetrate the skin. It occurs often after contact with cercariae containing water.
- Syncitium (pl. syncytia): A group of animal cells in which cytoplasmic continuity is maintained. Cytoplasmatic bridges join the cells together, but they remain discrete.

- Systematics: The study of the diversification of organisms and their natural relationship among living things through time. The relationships are visualized as evolutionary trees.
- Taxon: A unit group of one or more populations of organisms in the hierarchical classification.
- Taxonomy: The classical taxonomy is a study of defining groups of biological organisms on the basis of anatomical and morphological characteristics. Cytotaxonomy compares the size, shape and number of chromosomes of different organisms. Numerical taxonomy uses mathematical procedures to assess similarities and differences.
- Tegumentum: External body surface of trematodes and cestodes with a complicated structure. It functions as a nutrient absorption organ as well as a mechanical protection.
- Thymine: A pyrimidine nucleobase, also known as 5-methyluracil. Thymine is one of the four nucleobases in the nucleic acid of DNA.
- Trematoda: A class within the phylum of the parasitic flatworms (Platyhelminthes) containing the flukes such as Fasciola (liver fluke). Typical characteristics of flukes are the suckers and hooks to anchor themselves to the host. A cuticle protects the body surface of the flukes. Within a life-cycle the trematodes develop in one or more intermediate host, where the asexual reproduction takes place. In the definitive host the sexual reproduction occurs.
- Triclabendazole: An antihelmintic of the benzimidazole family. Benzimidazoles such as triclabendazole bind to beta-tubulin and prevent the polymerisation of the microtubules. It has a high efficacy against immature and adult liver flukes.
- Vector: Living carrier that transport the parasite in from one host to another.
- Ventral sucker: Also called acetabulum. Muscular, adhesive sucker. It is usually located near the mid-body of digenetic trematodes.
- Zoonosis: Animal infection that also may be transmitted to humans.

Definitions largely following:

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8. Appendix

8.1 Abstract

Molecular species determination of the prevalence of larval Digenea in aquatic snail species in the area of the river Leitha, from Götzendorf an der Leitha (lower Austria) to Potzneusiedl (Burgenland).

Digenean trematodes (= Digenea) comprise numerous species of medical and veterinary importance. They have complicated life cycles with molluscs serving as obligate first intermediate hosts, inhabiting sporocysts, rediae and cercariae. Often, those stages are morphologically indistinct and therefore not determinable at the species level.

The aim of this master thesis was to determine the occurrence of aquatic snails species and to identify their current infection rate with digenean trematodes in the area of the river Leitha, from 'Götzendorf an der Leitha' (Lower Austria) to 'Potzneusiedl' (Burgenland).

Species in focus of the study have been the invasive American Liver Fluke *Fascioloides magna*, as well as species of the family Schistosomatidae, parasites of birds, the cercariae of which may cause dermatitis in humans and animals.

In total, 1,184 samples (781 *G. truncatula*, 339 *L. stagnalis*, 63 *P. planorbis*, 1 *P. corneus*) of freshwater snails were collected. Of these, 66 individuals of *L. stagnalis*, 3 individuals of *P. planorbis* and 1 individual of *P. corneus* released cercariae. All of these individuals and 53 out of 781 individuals of *G. truncatula* were examined under the microscope for the presence of digenetic trematodes.

A total of 123 samples from affected snails were tested by a trematode universal PCR (Haider *et al.*, 2012). Out of these five samples were further tested by sequencing.

For the detection of *T. szidati*, *T. franki* and *T. regenti*, the schistosomatid cercariae (66 samples) were tested with a trichobilharzian specific PCR (Korsunen *et al.*, 2010). Furthermore, for species identification of the three most common European trichobilharzian species (*T. regenti*, *T. franki*, *T. szidati*) a PCR was chosen, that amplifies the region including ITS1, 5.8S rRNA and ITS2 sequences with the primer pair ITS5/ITS4. Altogether, 9 out of 66 tested samples were positive and were further tested with this PCR (Dvorak *et al.*, 2002). To sum it up, 7 out of 9 tested samples were positive in this PCR.

Altogether, 7 samples were further tested by sequencing after performing the ITS5/ITS4 PCR. By subsequent sequencing of the PCR products and alignment with reference sequences, a species-specific identification of the digenetic specimens was achieved.

In short, 83 trematode infections were detected in 1,184 examined *G. truncatula*, *L. stagnalis*, *P. planorbis* and *P. corneus*. The large American liver fluke *F. magna* as well as the common liver fluke *F. hepatica* was not found in the examined *G. truncatula*. But in three *L. stagnalis* *Trichobilharzia* spp. (prevalence 0.88%) were found. Additionally, in one *P. corneus* *B. polonica* was found.

Cercarial screenings are important to fill data in the databases. Only reliable databases of parasitic organisms warrant reliable epidemiological analyses, proper ecological evaluations, enhanced biodiversity data and significant medical risk assessments.

Zusammenfassung

Molekulare Artenbestimmung von larvalen digenen Trematoden in Süßwasserschnecken im Bereich der Ufer des Flußes Leitha, von Götzendorf an der Leitha (Niederösterreich) bis Potzneusiedl (Burgenland).

Digene Trematoden (=Digenea) beinhalten Arten von medizinischer und veterinärmedizinischer Bedeutung. Die Digenea verfügen über einen komplizierten Lebenszyklus und parasitieren als ersten obligaten Zwischenwirt Mollusken. In den Mollusken entwickeln sich verschiedene Larvenstadien, wie die Sporozysten, Redien und Zerkarien. Diese Larvenstadien sind oftmals mikroskopisch kaum unterscheidbar, was die Artunterscheidung erschwert.

Ziel dieser Arbeit war es, das Vorkommen und die Verbreitung von digenen Trematoden in verschiedenen Süßwasserschnecken in den Leitha-Auen, von Götzendorf an der Leitha (Niederösterreich) bis Potzneusiedl (Burgenland), zu ermitteln. Von März bis November 2012, wurden insgesamt 1.184 Süßwasserschnecken, darunter 781 *Galba truncatula*, 339 *Lymnaea stagnalis*, 63 *Planorbis planorbis* und 1 *Planorbarius corneus*, gesammelt. Insgesamt 66 *L. stagnalis*, 3 *P. planorbis* und 1 *P. corneus* setzten Zerkarien frei. Zusätzlich wurden 781 *G. truncatula* mikroskopisch auf das Vorhandensein digener Trematoden untersucht. Insgesamt wurden 123 isolierte Trematodenstadien mittels einer PCR (Haider *et al.*, 2012) molekularbiologisch auf

Trematoden überprüft. Danach wurden insgesamt 5 Proben mittels Sequenzierung überprüft.

Zur Identifizierung der *Trichobilharzia* Arten wurden die schistosomatiden Zerkarien mittels einer spezifischen PCR getestet (Korsunenکو *et al.*, 2010). Für die Identifikation von *T. szidati*, *T. franki* und *T. regenti* wurden die positiven Amplicons nochmal mittels einer PCR getestet (Dvorak *et al.*, 2002) und danach mittels Sequenzierung überprüft.

Weder der große amerikanische Leberegel *F. magna*, noch der gemeine Leberegel *F. hepatica* konnten in einer der untersuchten Schnecken detektiert werden. Allerdings wurden in drei *L. stagnalis* Trichobilharzien (3 *Trichobilharzia szidati*, 1 *Trichobilharzia* sp.) (Prävalenz: 0.88%) gefunden. Außerdem wurde in einer *P. corneus* *B. polonica* nachgewiesen.

Nur durch das kontinuierliche Überprüfen des Vorkommens von parasitären Organismen lassen sich zuverlässige epidemiologische Analysen, ordnungsgemäße ökologische Auswertungen, erweiterte Daten über Biodiversität und signifikante medizinische Beurteilung erstellen.

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9.5. Curriculum vitae

Personal information

Name: Larissa Gaub

Date and Place of Birth: 20th of January 1987 in Munich (Germany)

Nationality: Germany

Education

- June 2006: higher education entrance qualification (Pater-Rupert-Mayer Gymnasium, Munich, Germany)
- 2006 – 2007: Study of sociology (Kath. Universität Eichstätt)
- 2007 – 2010: Bachelor of Science: Biology (University of Regensburg)
- since March 2011: Master of Science, Molecular Biology (Universität of Vienna)
- since April 2012: Master Thesis at the Dep. of Medical Parasitology, Inst. of Specific Prophylaxis & Tropical Medicine, Medical University of Vienna in collaboration with the Natural History Museum Vienna, 3rd Zoological Dep.

Further Activities

- 2013/2014: Tutor at the Departement of Medical Parasitology, Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna

Attendance at national congresses 2012

- 46th Annual Meeting of the Austrian Society of Tropical Medicine and Parasitology, Innsbruck
- Nobis 6, Klagenfurt