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**Differences in the compatibility of infection between
the liver flukes *Fascioloides magna* and *Fasciola hepatica*
in a Colombian population of the snail *Galba* sp.**

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Running title: Compatibility of *Galba* sp. with two liver fluke species

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Abstract

Experimental infections of *Galba* sp. (origin, Colombia) with allopatric isolates of *Fasciola hepatica* from France or *Fascioloides magna* from Czech Republic were carried out during five successive snail generations to determine if this lymnaeid might sustain complete larval development of either parasite. In snails exposed to *F. hepatica*, 7 of 400 snails harboured several rediae and only 2 snails contained a small number of free cercariae on day 50 post-exposure. In contrast, the intensity of *F. magna* infection in *Galba* sp. progressively increased from the F1 to F5 generations. Spontaneous cercarial shedding of *F. magna* occurred in 7 of 100 *Galba* sp. belonging to the F5 generation and the number of shed cercariae did not significantly differ from that noted in control *Galba truncatula* of French origin. *Galba* sp. from Colombia can be added to the list of potential intermediate hosts for *F. magna*.

Key words: cercaria, experimental infection, *Fasciola hepatica*, *Fascioloides magna*, *Galba* sp., *Galba truncatula*, redia, snail generation.

Introduction

Freshwater snails of the Lymnaeidae family are known to be intermediate hosts for many digenetic species. According to Brown (1978) and Mas-Coma *et al.* (2005), members within this family can sustain larval development of more than 70 different trematodes. As epidemiological studies made on these parasites need the accurate identification of the snail host, several methods using shell morphology and/or the anatomy of the reproductive system have been proposed over the years to classify these lymnaeids (Hubendick, 1951; Burch, 1982).

Shell characters have been extensively, and very often exclusively, used to build the systematics of molluscs. Marine shells are diversified and present a very large set of morphological characters, i.e. protoconch structure, teleoconch ornamentation, radula morphology, etc..., that have proved useful in malacological taxonomy. In freshwater pulmonates, these shell characters are for the most part absent and shell shape only has been used to describe new taxa in the last 19th and 20th centuries and even sometimes in most recent years. As a result of using shell as diagnostic species character, a plethora of names may be found in the literature on freshwater pulmonates, numerous probably being synonyms. The case of the Lymnaeidae family is caricatural with about 1200 described species and several dozens of genera (Hubendick, 1951; Burch, 1982), whereas recent studies suggested that the family contained approximately 100 species (Strong *et al.*, 2008; Jarne *et al.*, 2010).

The classification systems based on morphological characters of shell shape have rapidly generated a controversy because lymnaeids exhibited a great diversity in shell morphology linked to substantial eco-phenotypic plasticity (Samadi *et al.*, 2000; Hurtrez-Boussès *et al.*, 2005; Schniebs *et al.*, 2011). It is accepted that anatomical characteristics of the snail's reproductive system are more homogeneous (Pointier *et al.*, 2009; Correa *et al.*, 2011), but only DNA-based analyses, i.e. phylogeny and barcoding, developed from the 2000s could effectively solve this taxonomic problem. As these last analyses allowed to ascribe individuals to one species or another, several new species such as *Lymnaea neotropica* (Bargues *et al.*, 2007), *Galba* sp. (Correa *et al.*, 2010, 2011) or *Lymnaea schirazensis* (Bargues *et al.*, 2011) have been recognized and/or described.

The conchological characteristics of *Galba* sp. correspond to that of several dozens of taxa reported in North America, presumably the geographic origin of this small-shelled species clade (see Hubendick, 1951; Burch, 1982; Correa *et al.*, 2010; Johnson *et al.*, 2013). This snail was arbitrarily identified by Bargues *et al.* (2011) as *L. schirazensis*, a species originally described from Shiraz by Küster in 1862. However, the original description of *L. schirazensis* is only based on conchological characters, i.e. three lines in Latin and 16 lines in German (see von den Busch in Küster, 1862), that have proved inadequate to ascribe any sampled lymnaeid to a given species

(Samadi *et al.*, 2000; Correa *et al.*, 2011). *Galba* sp. was reported also by Correa *et al.* (2010, 2011) as sufficiently divergent from other *Galba* species to be considered as a different species. Malacological surveys and subsequent molecular characterization revealed the occurrence of this taxon in the Dominican Republic, Ecuador, Egypt, Iran, Mexico and Peru (as *Lymnaea schirazensis*: Bargues *et al.*, 2011) as well as in La Reunion Island, Spain and Venezuela (as *Galba* sp.: Correa *et al.*, 2011). These two studies (Bargues *et al.*, 2011, and Correa *et al.*, 2011) clearly demonstrated that this taxon is an overlooked highly invasive species.

The snail *Galba* sp. has apparently a worldwide distribution (Correa *et al.*, 2010, 2011; Bargues *et al.*, 2011), which raises the question of whether this snail is able to sustain larval development of different digeneans. Among them, the liver fluke, *Fasciola hepatica*, is the most known (Torgerson & Claxton, 1999; Hurtrez-Boussès *et al.*, 2001) and some recent papers have mentioned natural or experimental infection of South American lymnaeids with this parasite (Pointier *et al.*, 2006, 2009; Bargues *et al.*, 2007, 2012a, b; Mera y Sierra *et al.*, 2009). Even if the presence of another digenean, *Fascioloides magna*, was not still reported in South America, a recent paper (Sanabria *et al.*, 2013) reported successful experimental infections of *Lymnaea neotropica* and *L. viatrix* var. *ventricosa* with this parasite. In view of the above results, the following two questions arose: might a Colombian population of *Galba* sp. sustain complete larval development of *F. hepatica* with cercarial shedding? Was also this population a potential intermediate host for *F. magna*? To answer these questions, five successive generations of this snail were experimentally infected with *F. hepatica* or *F. magna* to determine if this species is a potential intermediate host for these digeneans.

Materials and methods

Collection of snails and fluke eggs

The population of *Galba* sp. was collected from a farm at Finca el Ayer (6°28'9" N, 75°22'27" W) at altitude of 2,248 m near Medellin, department of Antioquia, Colombia. The population as *Galba* sp. was identified as *Galba* sp. via the study of rDNA (18S, ITS-1, ITS-2) and mtDNA *cox1* because the sequences obtained were similar to those deposited in GenBank by Bargues *et al.* (2011) and Correa *et al.* (2011). A total of 28 adult snails were collected from this farm in May 2012 and were raised in 14-cm Petri dishes at 20°C under laboratory conditions according to Rondelaud *et al.* (2007). Egg masses laid by these parent snails were collected and placed into other 14-cm Petri dishes containing oxygenated spring water and finely powdered lettuce as food for newly hatched individuals. Those, which attained 4 mm in shell height, were considered as the F1 generation and were used for experiments. The protocol was similar for the F2, F3, F4 and F5 snail generations (see below). A

total of 700 snails, measuring 4 ± 0.1 mm in height and belonging to the F1, F2, F3, F4 and F5 generations were used for experiments (Table 1). To compare the characteristics of *F. magna* infection in *Galba* sp. with those found in a common intermediate host of this parasite, a wild population of *Galba truncatula* was selected. These snails colonized a road ditch at Chézeau Chrétien ($46^{\circ}40'27''$ N, $1^{\circ}21'21''$ E), commune of Chitray, department of Indre, central France. One hundred snails (shell height, 4 mm), belonging to the overwintering generation, were collected from this population and acclimatized for 24 hours to laboratory temperature before being directly exposed to *F. magna* miracidia (Table 1).

Eggs of *F. hepatica* came from the gall bladders of naturally infected cattle at the slaughterhouse of Limoges, department of Haute Vienne, central France. Those of *F. magna* were collected from adult flukes recovered from the livers of naturally infected red deer (*Cervus elaphus*) hunted near the Mirošov village, Central Bohemia, Czech Republic. These egg isolates were washed several times with spring water and were incubated for 20 days at 20°C in the dark (Ollerenshaw, 1971).

Experiment 1: Galba sp. as a potential host for *Fasciola hepatica* & *Fascioloides magna*

The susceptibility of *Galba* sp. to *F. hepatica* or *F. magna* miracidia was studied in five successive snail generations via an experimental protocol already used by Sanabria *et al.* (2012) for *F. hepatica* and Vignoles *et al.* (2014) for *F. magna*. F2 snails originated from eggs laid by parasite-exposed individuals of the F1 generation between weeks 2 and 5 post-exposure. A similar protocol was used for the F3, F4 and F5 generations. This protocol was chosen in order that these descendants have a first (F2) or multiple contacts (F3, F4 or F5 generations) with the parasite through their parents.

The aim of the first experiment was to determine the aptitude of *Galba* sp. as a snail host for *F. hepatica* or *F. magna*. Five groups of *Galba* sp. with a variable number of snails per group were constituted for infections with *F. hepatica* (Table 1). In addition, four other groups of 50 snails each were constituted for the F1, F2, F3 or F4 generations of *Galba* sp. and were exposed to *F. magna* miracidia (Table 1). Each snail was subjected to two miracidia of *F. hepatica* or a single miracidium of *F. magna* for four hours at 20°C in 3.5 mL spring water. The choice of this sequence in the number of miracidia per snail was based on our experience in the production of trematode infective stages (Vignoles *et al.*, 2006; Rondelaud *et al.*, 2014a). Snails were then raised for 50 days in groups of ten individuals in 14-cm Petri dishes with 60 mL spring water per dish according to the method by Rondelaud *et al.* (2007). Snail food consisted of dried leaves of pesticide-free lettuce and dead *Molinia caerulea* leaves, while stems of live *Fontinalis* sp. ensured oxygenation of the water layer. Dissolved calcium in spring

water was 35 mg/L. Petri dishes were placed in an air-conditioned room under the following conditions: a temperature of 20°C, natural photoperiod of ten hours light. On day 50 post-exposure, surviving snails were dissected under a stereomicroscope to detect the presence of larval forms of *F. hepatica* or *F. magna* within their bodies and determine the most developed stage: immature rediae, cercariae-containing rediae, or free cercariae. Infected snails were counted taking into account snail generation and each developmental stage of larval development.

Experiment 2: comparison of Fascioloides magna infection in Galba sp and G. truncatula

As numerous free cercariae exceeding 20 per snail were only noted in the F3 and F4 generations of *F. magna*-infected *Galba* sp. (Table 1), a second experiment was carried out to determine the characteristics of these *F. magna* infections in snails belonging to the F5 generation and compare them with those which occurred in *G. truncatula*. One hundred *Galba* sp. and one hundred *G. truncatula* were used (Table 2). Snail exposure to miracidia and maintenance during the first 30 days were similar to those in the first experiment. On day 30, each surviving snail was isolated in a 35-mm Petri dish with pieces of dead grass, lettuce and spring moss, and placed at 20°C. Spring water and food were changed, if necessary, every day until snail death. When the first cercarial shedding occurred, surviving snails were subjected to a thermal shock every three days by placing their Petri dishes at 10°C-13°C for 3 hours to stimulate cercarial exit (Sanabria *et al.*, 2012). After their emergence, cercariae were counted and removed from Petri dishes. At the death of each infected snail, its shell was measured using callipers. Cadavers of non-shedding snails were routinely dissected under a stereomicroscope to count free rediae and free cercariae.

Data analysis

The first two parameters were snail survival on day 30 post-exposure and the prevalence of *F. hepatica* or *F. magna* infection calculated in relation to the number of snails surviving on day 30. In the first experiment, prevalence took into account the numbers of snails with immature rediae only, cercariae-containing rediae, or with free cercariae. In the second experiment, cercariae-shedding and non-shedding snails were used for prevalence calculation. For each parameter, the differences were analyzed using a χ^2 test. In the second experiment, shell growth of infected snails during the experiment, the length of pre-patent and patent periods, and the total number of shed cercariae were also calculated. Free rediae and free cercariae counted in the cadavers of non-shedding snails were also considered. Individual values recorded for these last six measurements

were averaged and their standard deviations were established for each snail group. One-way analysis of variance (ANOVA) was used to establish levels of statistical significance. The different analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Experiment 1: Galba sp. as a potential host for Fasciola hepatica & Fascioloides magna

Survival rates of *F. hepatica*-exposed *Galba* sp. on day 30 post-exposure ranged from 18.6% to 34.0% although no significant difference between these rates was found (Table 1). Low prevalences of <16% were noted in the F2-F5 generations of snails, without significant difference. However, larval development of *F. hepatica* was not complete in these snails because two individuals only harboured immature rediae, three several rediae containing a few differentiating cercariae and the last two others a few rediae and several free cercariae within their bodies. The number of free rediae ranged from three to six, while the numbers of free cercariae in the two snails of the F2 and F3 generations were 7 and 14, respectively (data not shown). In *Galba* sp. exposed to *F. magna*, the survival rate on day 30 was not significantly different from the F1 to F4 generations (Table 1). A similar finding was also noted for prevalence of *F. magna* infection through each snail generation. In spite of this, the intensity of snail infection increased with increasing snail generation, ranging from two snails harbouring immature rediae in the F1 to nine snails in the F4 generation. In this last generation, 5 of 9 individuals contained free cercariae.

Experiment 2: comparison of Fascioloides magna infection in Galba sp and G. truncatula

Survival of *Galba* sp. on day 30 post-exposure was significantly lower than for *G. truncatula* (Table 2). Similar findings were also noted for prevalence of *F. magna* infection and shell growth of infected snails during the experiment. Comparison of the pre-patent periods, patent periods or the numbers of shed cercariae for both snail species revealed no significant differences. Spontaneous cercarial shedding occurred in 4 of 7 *Galba* sp., with one, two or three shedding waves for 1, 2 and 4 snails, respectively (data not shown). In *G. truncatula*, cercarial shedding occurred always after a thermal shock and one, two, three or four shedding waves were noted for 9, 7, 9 and 14 snails, respectively (data not shown). In non-shedding *Galba* sp., the number of free rediae was significantly lower than that noted in non-shedding *G. truncatula*, while the number of free cercariae did not significantly differ from each other.

Discussion

Low survival rates on day 30 post-exposure were noted in the ten groups of *Galba* sp., whatever the digenean species (Tables 1 and 2). These findings may be mainly explained by the negative effect of the parasite species used on the life span of the snail. However, another hypothesis, based on inadequate conditions for *Galba* sp. breeding in the laboratory, cannot be completely excluded. In the field, *Galba* sp. seems to be a more amphibious snail than *G. truncatula* because adult individuals of *Galba* sp. were often observed on the emerged soil up to a 2-m distance from the waterside.

In spite of five successive generations of *Galba* sp. exposed to *F. hepatica* miracidia, only a few snails contained several rediae with/without free cercariae in their body on day 50 post-exposure. This finding cannot be explained by a low infectivity of French *F. hepatica* miracidia used in the present study because these larvae, originating mainly from local triclabendazole-treated cattle, showed a strong infectivity since the 2000s (Dreyfuss *et al.*, 2007) and experimental infections of local *G. truncatula* resulted in 72.1% prevalence with 44 cercariae-shedding snails out of 61 individuals surviving on day 30 and in a mean production of 222.1 shed cercariae (Sanabria *et al.*, 2012). It cannot also be explained by a low development of parasite larval forms over time because a few free cercariae were noted in the body of two dissected *Galba* sp. on day 50. Under these conditions, it is necessary to admit a problem of compatibility between *Galba* sp. and the French isolate of *F. hepatica*. This finding was surprising because our snails originated from the department of Antioquia (Colombia) and this district was known to be an at-risk district for human and animal fasciolosis (Mas-Coma *et al.*, 2009; Correa *et al.*, 2011; Valencia-López *et al.*, 2012). However, *Galba* sp. was not completely refractory to the parasite because of development of *F. hepatica* larval forms in several experienced snails (Table 1). A similar result was obtained for *L. schirazensis* using an isolate of *F. hepatica* from Poland and a snail strain from Spain (Bargues *et al.*, 2011). To explain the problem of compatibility between *Galba* sp. and the parasite, three hypotheses may be proposed. First, the compatibility between *Galba* sp. and *F. hepatica* might need strong selection process among numerous successive generations of snails, as that suggested by Boray (1969) in his review on fasciolosis for several lymnaeid species. Secondly, a snail co-infection with *F. hepatica* and another digenean such as a paramphistomid might be necessary to induce and favour larval development of *F. hepatica*, as demonstrated by Southgate *et al.* (1989) in the model *Bulinus globosus-Schistosoma bovis* and Abrous *et al.* (1998, 1999) in the model *Omphiscola glabra-F. hepatica*. Thirdly, variation in compatibility between strains of snails and geographic isolates of parasites may occur. Such a strong variation has already been shown in Cuba using different combinations of *F. hepatica* geographic isolates and populations of *Galba cubensis* and

Pseudosuccinea columella from different localities (Vázquez *et al.*, 2014). In this study, infection rates of *G. cubensis* may vary from 0 to 100% for the Sagua and Arroz isolates of *F. hepatica*, respectively.

After five generations with selection of infected snails, we obtained complete larval development of *F. magna* under experimental conditions, even if the infection of these five successive snail generations was necessary to have a progressive increase in the intensity of *F. magna* infection. This finding suggests a progressive and rapid adaptation of this snail population to the parasite through several successive snail generations. The present results do not bring any substantial element in favour of a particular susceptibility of Central and South American *Galba* species to *F. magna*. In our opinion, this successful infection of the Colombian *Galba* sp. would only result from the sole infectivity of the miracidium. An argument supporting this hypothesis is the wide range of intermediate hosts reported in North America (Dunkel *et al.*, 1996) and Europe (Huňová *et al.*, 2012; Novobilský *et al.*, 2012; Rondelaud *et al.*, 2014b) for this digenean.

Results for the F5 generation of *Galba* sp. and *F. magna* are inconclusive. Even if snail survival on day 30 and prevalence of *F. magna* infection were significantly lower than those noted for *G. truncatula*, the pre-patent period and the number of shed cercariae did not significantly differ between *Galba* sp. and *G. truncatula*, whereas the shell growth of *Galba* sp. during the experiment was lower: 1.7 mm instead of 3.5 mm for *G. truncatula* (Table 2). Moreover, spontaneous cercarial shedding of *F. magna* occurred in 4 of 7 snails, whereas this process was scarce for *G. truncatula* under laboratory conditions without thermal shock (Erhardová-Kotrlá, 1971; Vignoles *et al.*, 2006). In spite of differences in snail survival and prevalence of infection, *Galba* sp. seems to be a suitable intermediate host like *G. truncatula*. No reliable explanation can be given at the present time to explain the performances of this Colombian population of *Galba* sp. in larval development of *F. magna* and additional studies are still necessary to verify if other populations of this taxon can also sustain complete larval development of this digenean with the same performances.

In conclusion, *Galba* sp. can be added to the list of potential intermediate hosts of *F. magna* as this species was experimentally infected. Although the susceptibility of this species to infection with French miracidia of *F. hepatica* was very low, we noted that it was not totally refractory. Additional studies on other populations of *Galba* sp. are still necessary to determine if the low susceptibility to French *F. hepatica* and the good aptitude for larval development of *F. magna* only concern the Colombian population used in the present study, or if they can be generalized to other haplotypes of this species living in the Old and New Worlds (Correa *et al.*, 2011).

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Table 1

Characteristics of infections in several generations of *Galba* sp. exposed to *Fasciola hepatica* and *Fascioloides magna* on day 50 (first experiment). N = number of exposed snails on day 1.

Fasciolid species and snail generation	N	Snail survival (%) on day 30 post-exposure	No. of infected snails with			Overall prevalence (%)
			Immature rediae	Cercariae- containing rediae	Free cercariae rediae	
<i>F. hepatica</i>						
F1	50	26.0				0
F2	100	20.0	1		1	10.0
F3	150	18.6		1	1	7.1
F4	50	26.0	1	1		15.3
F5	50	34.0		1		5.8
<i>F. magna</i>						
F1	50	22.0	2			18.1
F2	50	30.0	1	2		20.0
F3	50	34.0	1	3	2	35.2
F4	50	46.0	2	2	5	39.1

Table 2

Characteristics of *Fascioloides magna* infection in the F5 generation of *Galba* sp. and in *Galba truncatula* subjected to single-miracidium infections and reared at 20°C (second experiment). Mean values of six parameters are given ± standard deviations and levels of significance include * p<0.05, ** p<0.01, *** p<0.001.

Population	<i>Galba</i> sp., F5	<i>G. truncatula</i>
Snail survival (%):		
- Day 1	100	100
- Day 30	48.0	71.0**
Number of snails:		
- cercariae-shedding snails	7	39
- non-shedding snails	4	15
Prevalence of <i>F. magna</i> infection (%)	22.9	76.0***
Growth of cercariae-shedding and non-shedding snails (mm)	1.7 ± 0.5	3.5 ± 0.6***
Pre-patent period (days)	67.4 ± 9.3	69.8 ± 5.7
Patent period (days)	16.2 ± 3.7	22.5 ± 5.8
Number of shed cercariae	96.3 ± 27.4	119.4 ± 33.1
Cadavers of non-shedding snails:		
- Free rediae	24.3 ± 5.7	34.8 ± 7.5*
- Free cercariae	157.8 ± 34.7	196.0 ± 41.1