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RESEARCH NOTE

Lymnaea cubensis, an experimental intermediate host for *Fascioloides magna*

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Abstract: Single-miracidium infections of *Lymnaea cubensis* (Pfeiffer) from Guadeloupe with the giant liver fluke *Fascioloides magna* (Bassi, 1875) (Digenea) were carried out during five successive snail generations to determine if this lymnaeid might sustain complete larval development of the parasite. Controls were constituted by a French population of *Galba truncatula* (Müller) (a single generation) infected according to the same protocol. It was recorded that prevalence and intensity of *F. magna* infection in *L. cubensis* progressively increased from F1 to F5 generations. Cercarial shedding of *F. magna* was noted only within F5 generation of *L. cubensis.* However, most measured parameters of infection in this species were significantly lower than those noted for *G. truncatula* and most *L. cubensis* died after a single shedding wave. Despite this, *L. cubensis* can be added to the list of potential intermediate hosts of *F. magna*.

Keywords: giant liver fluke, experimental infections, *Galba truncatula*, snail generation, snail susceptibility

The success of a snail infection by a given digenean (Digenea) mainly depends on the susceptibility of this snail as an intermediate host (an interpopulation variability in susceptibility exists for many snail species) and the infectivity of miracidia (Smyth and Halton 1983). If a snail species other than the common snail host is used for infection, the process often results in abortive infections, but sometimes it may lead to development of infections with an increased production of cercariae.

In the case of the liver fluke, *Fasciola hepatica* Linnaeus, 1758, three South American lymnaeids, *Lymnaea neotropica* Bargues, Artigas, Mera y Sierra, Pointier et Mas-Coma, *L. viatrix* var. *ventricosa* d'Orbigny and *L. cubensis* (Pfeiffer) are known to sustain larval development of *F. hepatica* – see Morales and Pino (1983) and Bargues et al. (2007). Two of these snail species, *L. neotropica* and *L. v. ventricosa*, can also ensure larval development of the giant liver fluke *Fascioloides magna* (Bassi, 1875) (Digenea: Fasciolidae) (Sanabria et al. 2013), which is relatively close to *F. hepatica*.

This finding implicated the question as to whether other semiaquatic lymnaeids living in the New World such as *L. cubensis* would also sustain complete larval development of *F. magna*. This assumption is supported by the fact that *L. cubensis* is morphologically similar and genetically close to *L. neotropica* and *L. v. ventricosa* – Correa et al. (2010, 2011), and that it is susceptible to *F. hepatica* infection (Morales and Pino 1983, Bargues et al. 2007). Therefore, we followed the former experiments with *L. neotropica*/*L. v. ventricosa* and *F. hepatica*/*F. magna* and performed the additional experimental infections of a Guadeloupean population of *L. cubensis* with *F. magna*.

This lymnaeid was selected as a model for the following reasons: (i) *F. magna* was known to be enzootic in the south-eastern part of North America, i.e. lower Mississippi and around the coast of the Gulf of Mexico (Pybus 2001); (ii) the geographical distribution of *L. cubensis* covers the southeastern part of North America, the Caribbean and the northern part of South America (Dillon et al. 2006); (iii) a case of an imported wapiti was reported in Cuba (Lorenzo et al. 1989); and (iv) since there is evidence of significantly overlapping geographical distributions of the parasite *F. magna* and the snail *L. cubensis*, the susceptibility of this lymnaeid to *F. magna* infection and its potential role in transmission of fascioloidosis have to be tested.

Single-miracidium infections of this species with *F. magna* were thus carried out during five snail generations (from F1 to F5) to determine if larval development of this parasite can be complete in this lymnaeid and, if so, to specify the characteristics of snail infections. French *Galba truncatula* (Müller) infected during a single snail generation with the same miracidial isolate and raised according to the same protocol served as control.

The population of *L. cubensis* was living in a swampy zone bordering a mangrove at Pico (16°21'13"N; 61°27'40"W), commune of Morne-à-l'eau in Guadeloupe (French West Indies). Adult snails were collected in February 2012 before being raised in the laboratory at 22–24 °C according to the method by Rondelaud et al. (2007). Five successive snail generations (F1–F5) of this species were used for experimental infections. The population of *G. truncatula* colonized a road ditch at Chézeau Chrétien (46°40'27"N; 1°21'21"E), commune of Chitray, department of

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Table 1. Characteristics of *Fascioloides magna* infection in four generations of *Lymnaea cubensis* subjected to single-miracidium infections, raised at 22–24 °C and dissected at day 50 p.e. (post exposure; experiment A).

	Generation of *Lymnaea cubensis*			
Parameters	F1	F2	F3	F4
Number of snails				
- at exposure	50	50	50	50
- at day 50 p.e. (rate in %)	18 (36)	25 (50)	29 (58)	27 (54)
Number of infected snails				
- with immature rediae	-	-	-	10
- with cercariae-containing rediae	-	7	9	5
- with free cercariae	3	1	3	2
Overall prevalence of infection (%)	17	35	41	63

Indre, central France. This population was already used for experimental infections with *F. magna* by Sanabria et al. (2013).

Fifty snails (shell height 4 mm) for the F1, F2, F3 or F4 generations of *L. cubensis* (Table 1), 100 for the F5 generation and 100 for *G. truncatula* (Table 2) were collected from each population. Eggs of *F. magna* were collected from adult flukes recovered from the livers of naturally infected red deer (*Cervus elaphus* Linnaeus) hunted near the village of Mirošov, Central Bohemia, Czech Republic. The eggs were washed several times with spring water and were incubated for 20 days at 20 °C in the dark (Ollerenshaw 1971).

The susceptibility of *L. cubensis* to *F. magna* miracidia was studied during five successive snail generations (from F1 to F5) via an experimental protocol already used by Sanabria et al. (2012) for *F. hepatica*. The F2 snails originated from eggs laid by infected individuals of F1 generation between weeks 2 and 5 post-exposure (p.e.). A similar protocol was used for F3, F4 and F5 generations. This protocol was chosen because these descendants had a first contact (F2) or multiple (F3–F5 generations) contacts with the parasite through their infected parents.

Two experiments, A and B, were carried out. Four groups of 50 snails each (one group per snail generation from F1 to F4, Table 1) were constituted in experiment A. Single-miracidium infections were performed for each snail for 4 hours at 20 °C in 3.5 ml of spring water. Snails were then raised in groups of 10 individuals in 14 cm Petri dishes (volume of spring water, 60 ml) for 50 days according to the method by Rondelaud et al. (2007). Snail food consisted of dried leaves of lettuce and dead leaves of *Molinia caerulea* (Linnaeus), whereas several 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 22–24 °C, natural photoperiod of 10 hours light. At day 50 p.e., the surviving *L. cubensis* were dissected under a stereomicroscope to detect the presence of *F. magna* larval forms within their bodies and determine the most developed stage (immature rediae, cercariae-containing rediae, or free cercariae). Infected snails were then counted taking into account snail generation and each developmental stage of larval development.

The aim of experiment B was to follow the dynamics of *F. magna* cercarial shedding in experimentally infected snails until their natural death. One hundred snails belonging to F5 generation of *L. cubensis* and 100 *G. truncatula* were used. The shell height of these snails was measured using callipers just before miracidial exposure to use 4 ± 0.1 mm high individuals only. Snail exposure to miracidia and snail breeding during the first 30 days p.e. were similar to those in experiment A. At day 30, each surviving snail was isolated in a 35-mm Petri dish to easily count metacercariae on dish bottom and walls during the patent period. Pieces of dead grass, lettuce and spring moss were put in these 35-mm dishes, which were also placed at 22–24 °C. A daily surveillance was made to change spring water and food if necessary. As cercarial shedding of *F. magna* from experimentally-infected snails under laboratory conditions was sporadically reported (Erhardová-Kotrlá 1971, Vignoles et al. 2006), the surviving snails were subjected to a thermal shock to stimulate cercarial exit when the first cercarial shedding occurred.

To avoid a possible too high mortality of *L. cubensis* infected with *F. magna*, the temperature used for inducing the thermal shock was chosen according to temperature range prevailing in the natural habitat of *L. cubensis*. Petri dishes containing surviving snails were thus placed at 15–18 °C for 3 hours according to the indications by Rondelaud et al. (2013) and Sanabria et al. (2013). After the replacement of Petri dishes at 22–24 °C, cercariae exited from the snails in the following 2–3 hours and metacercariae were counted before their removal from Petri dishes. At the death of each infected snail (between days 81 and 95 p.e. for *L. cubensis*, and between days 82 and 99 p.e. for *G. truncatula*), its shell was again measured using callipers. Cadavers of snails containing cercariae but without shedding (NCS snails) were then routinely dissected under a stereomicroscope to count free rediae and free cercariae.

The first parameter calculated was snail survival at day 50 p.e. (date of snail dissection) in experiment A or at day 30 in experiment B. The choice of day 30 for experiment B was due to the fact that several *F. magna* cercariae may sometimes exit from infected snails before day 50 p.e. (D. Rondelaud, INSERM 1094, Faculties of Medicine and Pharmacy, Limoges, France – personal observation). Another parameter was prevalence of *F. magna* infection calculated in relation to the number of snails surviving at day 50 (A) or at day 30 (B). The numbers of snails with immature rediae only, cercariae-containing rediae, or with free cercariae (experiment A) and the quantities of cercariae-shedding snails (CS snails) and NCS snails dying after day 30 p.e. (experiment B) were concerned for prevalence calculation. For each parameter, the differences were analysed using a χ^2 test. In experiment B, the other parameters calculated were the shell growth of CS and NCS snails during the experiment (the difference between shell height at miracidial exposure and that measured at snail death), the length of the prepatent period for CS snails, that of the patent period, and the total number of metacercariae.

As cercarial shedding of *F. magna* was discontinuous during the patent period, with shedding waves (1–2 days each) separated by interwaves with no shedding (Vignoles et al. 2006), the number of CS snails showing a single or several shedding waves was considered. Lastly, free rediae and free cercariae counted in the cadavers of NCS snails were also taken into account. Individual values recorded for the shell growth of CS and NCS snails, the prepatent and patent periods, the total number of metacercariae, and the quantity of free rediae and free cercariae in NCS snails were averaged and their standard deviations were calculated considering snail groups. One-way analysis of variance (ANOVA) was used to establish levels of statistical signifi-

Table 2. Characteristics of *Fascioloides magna* infection in F5 generation of *Lymnaea cubensis* and in *Galba truncatula* subjected to single-miracidium infections and raised at 22–24 °C (experiment B).

Parameters	*L. cubensis*, F5	*G. truncatula*	Statistics
Number of snails			
- at exposure	100	100	-
- at day 30 p.e. (rate in %)	54 (54)	74 (74)	$\chi^2 = 8.68$, $P < 0.01$
Number of snails after day 30 p.e.			
- CS	8	41	-
- NCS	7	11	-
- Uninfected	39	18	-
Prevalence of infection (%)	28	70	$\chi^2 = 22.60$, $P < 0.001$
Shell growth (mm) during the experiment*			
- CS	2.8 (0.7)	3.5 (0.7)	$F = 4.21$, $P < 0.05$
- NCS	2.6 (0.8)	3.4 (0.6)	$F = 4.11$, $P < 0.05$
Prepatent period in days*	76.1 (13.5)	66.3 (7.1)	NS
Patent period in days*	14.2 (3.7)	24.0 (4.6)	$F = 11.07$, $F < 0.01$
Number of metacercariae*	61.0 (31.1)	107.9 (41.2)	$F = 4.05$, $P < 0.05$
Cadavers of NCS snails			
- Free rediae*	21.9 (5.2)	36.5 (6.2)	NS
- Free cercariae*	112.4 (23.2)	177.3 (35.0)	$F = 9.26$, $P < 0.01$

* mean value (SD); CS – cercariae-shedding snails; *F* – value of ANOVA; NCS – snails containing cercariae but without shedding; NS – not significant; P – probability; χ^2 – value of the χ^2 test.

Table 3. Numbers of cercariae-shedding (CS) snails belonging to *Lymnaea cubensis* (F5 generation) and *Galba truncatula* in relation to the number of shedding waves noted during the patent period (experiment B). n, total number of cercariae-shedding snails.

No. of shedding waves during the patent period	Number of CS snails	
	L. cubensis (n = 8)	*G. truncatula* (n = 41)
1	6	2
2	2	7
3	0	18
4	0	9
5	0	5

cance. Both types of analyses were calculated using the Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

Table 1 gives the numbers of infected snails in F1 to F4 generations infected with *F. magna*. The differences between survival rates at day 50 p.e. were insignificant, whatever the mode of comparison. In contrast, the number of snails with larval forms increased with increasing snail generation so that overall prevalence of infection was significantly greater ($\chi^2 = 10.13$, $P < 0.05$) in F4 generation than in the other generations. If three snails of the F1 generation harboured immature rediae, most differentiated larval forms were noted in the further generations, with presence of free cercariae in two F4 snails.

The characteristics of *F. magna* infection in F5 generation of *L. cubensis* and in *G. truncatula* are given in Table 2. Survival of *L. cubensis* at day 30 p.e. and prevalence of infection were significantly lower than for the other snail species. Similar findings were also noted for the shell growth of CS and NCS *L. cubensis* during the experiment. No significant difference between the prepatent periods was found, even if the mean length noted for *L. cubensis* (76 days) was higher. Compared to values noted for *G. truncatula*, significantly shorter patent periods and lower numbers of metacercariae were noted for *L. cubensis*. Compared to NCS *G. truncatula*, the number of free cercariae in the body of NCS *L. cubensis* was significantly lower, while the redial burdens did not significantly differ from each other.

Six CS *L. cubensis* (out of 8) died after a single shedding wave during the patent period (Table 3), while the death of most CS *G. truncatula* occurred after two (7 snails), three (18 individuals), four (9 snails) or five (5 individuals) shedding waves.

The Guadeloupean population of *L. cubensis* used in the present study was able of sustaining complete larval development of the parasite, including the cercarial shedding under experimental conditions. However, the infection of five successive generations of snails was necessary to have a progressive increase in prevalence and intensity of *F. magna* infection. This finding suggests a progressive adaptation of this population to the parasite through several successive snail generations, as already reported by Dreyfuss et al. (2010) for *Lymnaea glabra* Müller. This progressive adaptation of *L. cubensis* to the parasite might be related to a change in the host immune response, which will gradually remove the bottleneck exerted by the snail body on parasite development. This hypothesis is supported by the work of Szmidt-Adjidé et al. (1996) on infected *G. truncatula*. According to these authors, many neurons of the cerebroid and pedal ganglia would degenerate and lost the function during snail infection. According to this assumption, the ganglia of *L. cubensis* would secrete a lower and lower amount of neuromediators through successive generations of infected snails and thus induce a progressive change in the mechanisms of immune system response to the parasite and a progressive lifting of the bottleneck exerted by the snail on larval development of the parasite. However, a decrease in *L. cubensis* resistance due to the conditions of snail breeding in the laboratory cannot be completely excluded.

Compared to control *G. truncatula*, most characteristics of infection in F5 generation of *L. cubensis* (Table 2) were significantly lower. The most surprising point was the low shell growth of CS and NCS *L. cubensis* during snail infection (2.6–2.8 mm, Table 2), whereas the corresponding shell growth of CS and NCS *G. truncatula* was 3.4–3.5 mm. To explain this difference, it is necessary to realize that breeding conditions used to raise *G. truncatula* in the laboratory (Rondelaud et al. 2007, 2009) were not probably absolutely ideal for *L. cubensis* and that the particular elements (possibly a kind of nutrition that the lymnaeid meets in its natural habitat) may have been lacking in this breeding protocol.

In experiment B, the number of cercariae shed by CS *L. cubensis* and that of free cercariae present in NCS *L. cubensis* were also significantly lower than those noted for control *G. truncatula*. These two findings in *L. cubensis* can be explained by the lower shell growth of these infected snails during the experiment and, consequently, by the lower number of free rediae that developed within their bodies. Indeed, it is well known that a positive relationship between the shell growth of infected snails and cercarial production exists in some snail-parasite models, as demonstrated by Zischke (1967) for *Echinostoma revolutum* (Frölich, 1802) or by Rondelaud and Barthe (1987) for *F. hepatica*. However, in spite of these differences, *L. cubensis* seems to be suitable intermediate host for larval development of *F. magna*, similarly to *L. v. ventricosa* – Sanabria et al. (2013).

Cercariae from six CS *L. cubensis* (out of 8) were shed during a single wave just before the death of snails (Table 3), whereas cercariae from 39 CS *G. truncatula* (out of 41) were released during 2–5 shedding waves. These findings can also be explained by the above hypothesis of a progressive adaptation of this *L. cubensis* population to *F. magna.* An argument supporting this assumption is the occurrence of numerous *F. hepatica* cercarial shedding waves in the case of *G. truncatula*, the dominant snail host in Europe for *F. hepatica* (see Dreyfuss and Rondelaud 1994), whereas there are some records of lower number of shedding waves in the case of unusual snail hosts such as *L. glabra* or *Planorbis leucostoma* Kozkov (see Abrous et al. 1998). In conclusion, since *L. cubensis* was successfully experimentally infected, it can be added to the list of potential intermediate host of *F. magna*.

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