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Growth, Laying, and Survival Rates of the *Galba truncatula* Snails Infected with *Fasciola hepatica*

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ABSTRACT

Fasciolosis is one of the most important parasitic diseases in ruminants in Algeria, of which the intermediate host is *Galba truncatula* (*G. truncatula*) snail. The current study aimed to investigate the prevalence of *Fasciola* sp. in naturally infected *G. truncatula* snails using multiplex PCR. Secondly, it was targeted toward examining the rate of growth, survival, and laying of the snails in experimental conditions during 6 weeks of rearing in three tanks. This study was conducted in two different regions of Algeria, namely El Tarf and Constantine. The investigated tanks 1, 2, and 3 consisted of 12 (size 3-4 mm), 30 (size 5-6 mm), and 30 (size 7-8 mm) snails, respectively. *Fasciola* sp. DNA was detected in 33.33% of *G. truncatula* snails (25% in Constantine and 42.85% in El Tarf). The total survival rates in the first, second, and third tanks were 50%, 43.3%, and 40%, respectively. The obtained results indicated that the growth rate of the snail depended on its initial size (the smaller the initial size, the higher the weekly growth rate). The total growth rates were 3, 1.7, and 1.1 mm in tanks 1, 2, and 3, respectively. The use of multiplex PCR indicated a relatively high level of infestation of the snails by *Fasciola* sp. Snails larger than 7 mm had the highest lay rate. Further studies are needed to investigate other snails that may be infested with *Fasciola* sp.

Keywords: *Fasciola*, *Galba truncatula*, PCR multiplex, Snail

INTRODUCTION

Fasciola hepatica (*F. hepatica*, Linné 1758) is a zoonotic parasitic disease that affects mainly ruminants (Esteban et al., 2003). It has a complex life cycle with snails (Lymnaeidae) as intermediate hosts and ungulate mammals as final hosts (Špakulová et al., 2003). Fascioliasis represents a major economic problem by decreasing the productivity performance of animals due to the condemnation of affected organs (Reinaldo Gonzalez et al., 2002; Chauvin et al., 2007).

In Algeria, fasciolosis is one of the most important helminthic parasites of ruminants (Ouchene-Khelifi et al., 2018). In a study conducted in El Tarf, northeastern Algeria, the prevalence of fascioliasis infection was revealed at 26.7 ± 2.5%, 6.5 ± 0.4%, and 2.5 ± 0.2% in cattle, sheep, and goats, respectively (Ouchene-Khelifi et al., 2018). In Jijel, northern Algeria, the prevalence of fasciolosis was reported at 27.0% and 18.2% in cattle and sheep, respectively (Mekroud et al., 2004). The economic losses related to liver condemnation are very high and estimated at 8.2 million euros in Belgium (Charlier et al., 2009), 52 million euros in Switzerland (Schweizer et al., 2005), and 10000-60000 euros in Algeria (Mekroud et al., 2004; Ouchene-Khelifi et al., 2018). *Galba truncatula* (*G. truncatula)* is a lymnaeid snail intermediate host of *F. hepatica* (Torgerson and Claxton, 1999; Mekroud et al., 2002; Righi et al., 2016). It is a widespread species found mainly in periodically inundated sandy-muddy habitats as well as in running water (Vignoles et al., 2010; Rondelaud et al, 2014; Vignoles et al., 2017).

Detection of *Fasciola* sp. in snails is possible by microscopy or molecular techniques (Caron et al., 2014). Microscopic methods are fundamentally based on crushing, dissection of the snail, and/or cercarial excretion. Molecular detection can be efficient in screening naturally infected snails. Molecular analysis is done by using an internal control (multiplex PCR) to exclude the possibility of false-negative results (Caron et al., 2011; Caron et al., 2014).

In Algeria, there is a dearth of published data on the molecular prevalence of *Fasciola* sp. in *G. truncatula* snails. The aims of the present study were first to investigate the molecular prevalence of *Fasciola* sp. in naturally infected *G. truncatula* snails using multiplex PCR, and secondary, to study snails' growth and laying survival rates in El Tarf and Constantine, Algeria.

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MATERIALS AND METHODS

Ethical approval

The study was conducted based on the ethical rules of the Institute of Veterinary Sciences of Constantine University, Algeria.

Study region

The study was conducted in Algeria in two different regions of El Tarf and Constantine. El Tarf is characterized by clayey soil with moderate permeability, a humid and warm Mediterranean climate, and an average temperature of 19.6°C varying generally from 7°C to 32°C. The annual average rainfall is 550 mm. Constantine is characterized by a warm temperate climate. The average temperature is 15.6°C (ranges from 2°C to 34°C) and the average annual rainfall is 469 mm.

Molecular detection of *Fasciola hepatica* in *Galba truncatula* snails using multiplex PCR

A total of 32 snails were collected from typical biotopes on a perimeter of 1 m^2 in each biotope according to the recommendations of Mekoud et al. (2002). The snails were rinsed with water and then transported to the laboratory for molecular analysis. The size of all collected snails was measured before being analyzed by multiplex PCR. Of the 32 snails, 12 (mean size 4.3 mm) were collected from Constantine, and 20 (mean size 5.73 mm) were collected from El Tarf.

DNA extraction

Chelex® method was used for DNA extraction (Caron et al., 2011). Initially, the snails were disrupted by a pellet mixer (Trefflab) in 100 µl of Chelex® 5% (Biorad, Nazareth Eke, Belgium), then incubated for 1 hour at 56°C and 30 minutes at 95°C in a Peltier Thermal Cycler. The mixture was centrifuged (OHAUS Europe GmbH, Switzerland) at 13000 × g for 7 minutes. A spectrophotometer (Thermo Scientific, NanoDrop 1000) was used to measure DNA concentration and purity. The supernatant was collected and stored at -20°C until further analyses.

Multiplex PCR

The multiplex PCR was used according to a study by Caron et al. (2011) to amplify a highly repeated 124 bp sequence specific for *Fasciola* sp. (Kaplan et al., 1995) and ITS-2 rDNA sequence specific for lymnaeids (500–600 bp) (Bargues et al., 2001). For the amplification of *Fasciola* sp. sequences, the used primers were Fsh1 5′-GAT-CAA-TTC-ACC-CAT-TTC-CGT-TAG-TC C-TAC-3′ and Fsh2 5′-AAA-CTG-GGC-TTA-AAC-G GC-GTC-CTA-CGG-GCA-3′ and for lymnaeids, ITS-2 amplification were News2 5′-TGT-GTC-GAT-GAA-GA A-CGC-AG-3′ and Its2Rixo 5′-TTC-TAT-GCT-TAAATT-CAG-GGG-3′ (Almeyda-Artigas et al., 2000; Bargues et al., 2001).

The sequences were amplified using a commercial kit (Taq PCR Master Mix, Qiagen) in a total volume of 25 µl in a Peltier Thermal Cycler with an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles, each comprising denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes. After amplification, the electrophoresis was performed in agarose gel at 2% prepared in TAE buffer with ethidium bromide (myGel InstaView™, USA).

Collection of snails to study their growth, laying, and survival rates

The snails were collected from typical biotopes on a perimeter of 1 m^2 according to the recommendations of Mekroud et al. (2002). The snails were transported to the laboratory in special snail tanks at room temperature within 30 minutes. The allotment of the snails was done based on their size, on three rearing tanks. Tank 1 consisted of 12 snails (size 3-4 mm), tank 2 entailed 30 snails (size 5-6 mm), and tank 3 was filled with 30 snails (size 7-8 mm).

The rearing conditions were artificially ensured (water, oxygenation, lighting, and food) during a 6-week-follow-up period according to standardized *in vitro* rearing procedures (Rondelaud et al., 2002). The rearing tanks were checked daily and cleaned weekly. Food was distributed as needed (macerated salad), and the water level at the temperature of 22-25°C in the tanks was systematically checked (2 cm). The tanks were covered to avoid water evaporation and laying eggs of dipteran flies. Oxygenation of the water was ensured by a bubbler.

The snails were kept in culture for 6 weeks to study their survival rate, the growth rate of the shell, and some reproduction parameters of *G. truncatula*, including average number of embryos/ laying, average number of laying/limnea, and average number of embryos/limnea. The studied parameters and their mode of calculations are presented in Table 1.

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Table 1. Calculation method of the biological parameters studied in snails

Parameters	Expression
Weekly survival rate (relative) of snails in rearing tanks for 6 weeks (%)	(Number of live snails in the tank at the end of the week / Number of live snails at the beginning of the week) × 100 (%)
The total (absolute) survival rate of snails in rearing tanks for 6 weeks (%)	(Number of live slugs in the tank after 6 weeks/ Initial number of slugs put in the tank) x100 (%)
Average weekly growth rate (relative) of snails for 6 weeks	Average weekly snail size - Average snail size the previous week
Average growth rate (absolute) of the snails for 6 weeks	Average snail size in the last week - Average snail size in the first week
Total number of eggs for 6 weeks	Total number of eggs collected in the tank during the whole study period
Total number of embryos for 6 weeks	Total number of embryos counted in all clutches during the study period
Average number of eggs per snail	Average number of eggs collected/number of snails in the tank
Average number of embryos/ laying	Total number of embryos counted / Number of eggs harvested in the tank
Average number of embryos/ snail	Number of embryos counted/number of snails in the tank

RESULTS

Molecular study

The 32 collected snails were identified morphologically as *G. truncatula* and 15 of them were confirmed using multiplex PCR. Of the total snails, 17 (53.12%) were eliminated from the study due to PCR inhibition. The DNA of *Fasciola* sp. was detected in 5/15 snails (33.33%), of which 2/8 snails in Constantine and 3/7 snails in El Tarf, resulting in a prevalence of 25% and 42.85%, respectively.

Survival, growth, and laying rates of snails

The snails were identified morphologically as *G. truncatula*. The survival rates of snails were 61.9-100%, 70-100%, and 70.5-100% in tanks 2, 1, and 3, respectively. At the end of the experiment, the total survival rates were 40%, 43.3%, and 50% in tanks 3, 2, and 1, respectively (Table 2). The hebdomadal growth of the snails during 6 weeks in tanks 1, 2, and 3 were measured as 0-1.3 mm, 0-1.1 mm, and 0-0.6 mm, respectively (Table 3). The total growth of the snails during the study was 3 mm, 1.7 mm, and 1.1 mm in tanks 1, 2, and 3, respectively (Table 3). Regarding the laying rates, tank 1 included 29 eggs with 360 embryos (an average number of 30 embryos per snail). Tank 2 had 100 layings with 1851 embryos (61.7 embryos/snail), and tank 3 entailed 136 eggs with 2707 embryos with an average number of 90.23 embryos per snail (Table 4).

Table 2. Weekly and absolute survival rates of snails in the three tanks

	Tank 1		Tank 2		Tank 3	
Breeding weeks	**Initial number (mortality)**	**Survival rate**	**Initial number (mortality)**	**Survival rate**	**Initial number (mortality)**	**Survival rate**
First week	12 (00)	100%	30 (00)	100%	30 (00)	100%
Second week	12 (02)	83.3%	30 (06)	80%	30 (03)	90%
Third week	10 (03)	70%	24 (03)	87%	27 (05)	81.4%
Fourth week	07 (01)	82.7%	21 (00)	100%	22 (01)	95.4%
Fifth week	06 (00)	100%	21 (08)	61.9%	21 (04)	80.9%
Sixth week	06 (00)	100%	13 (00)	100%	17 (05)	70.5%
Total survival rate	06/12	50%	13/30	43.33%	12/30	40%

Table 3. Weekly and absolute growth of snails (mm/week) in the three tanks

Breeding weeks	Tank 1 (12 Snails)	Tank 2 (30 Snails)	Tank 3 (30 Snails)
First week	3.5 mm (--)	5.5 mm (--)	7.5 mm (--)
Second week	4.8 mm (1.3 mm)	6.6 mm (1.1 mm)	7.5 mm (00)
Third week	5.3 mm (0.5 mm)	6.9 mm (0.3 mm)	8.1 mm (0.6 mm)
Fourth week	6.3 mm (1 mm)	7.1 mm (0.2 mm)	8.2 mm (0.1 mm)
Fifth week	6.3 mm (00)	7.1 mm (00)	8.5 mm (0.3 mm)
Sixth week	6.5 mm (0.2 mm)	7.2 mm (0.1 mm)	8.6 mm (0.1 mm)
Total growth rate	3 mm	1.7 mm	1.1 mm

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Table 4. Characteristics of snail laying in the three tanks during 6 weeks of rearing

Parameters	Tank 1 (12 Snails)	Tank 2 (30 Snails)	Tank 3 (30 Snails)
Number of snails	12	30	30
Total number of laying's	29	100	136
Total number of embryos	360	1851	2707
Average number of laying per snail	2.42	3.33	4.53
Average number of embryos per laying	12.41	18.51	19.90
Average number of embryos per snail	30	61.7	90.23

DISCUSSION

Microscopy or molecular techniques are methods used for the detection of *F. hepatica* in snails (Caron et al., 2008). Molecular tools must be efficient enough to detect naturally infected snails (Caron et al., 2014). Molecular techniques have been used in several studies to investigate the prevalence of *F. hepatica* in experimentally infested snails, but a few studies have tested naturally infected snails (Relf et al., 2009; Kozak and Wedrychowicz, 2010; Martinez-Ibeas et al., 2013). In the current investigation, molecular investigation of *F. hepatica* DNA was performed in naturally infested *G. truncatula* snails.

In the present study, DNA extraction was employed using the Chelex® technique. This technique presents many advantages, including the ability to obtain amplifiable DNA in a rapid time, at a minimal cost, and without using toxic solvents and multiple tube transfers. The multiplex PCR was adapted to eliminate eventual PCR inhibitions (Gonzalez et al., 2004; Caron et al., 2008). However, in the present study, 17 samples of snails showed PCR inhibition. Using the internal control and the apparition of the ITS2 band, the snail parasite was deduced in contrast to other molecular techniques where false negatives cannot be excluded (Gonzalez et al., 2004; Caron et al., 2008).

In the current study, the proportion of PCR inhibition (53.12%) was relatively higher than the findings of Righi et al. (2016, 3.32%) and Caron et al. (2014, 7.89%). The eliminated snails can be contaminated by some PCR inhibitors, such as complex polysaccharides, humic acid, and proteinase (Caron et al., 2014). In the present study *Fasciola* sp. DNA was isolated by multiplex PCR from 33.33% of *G. truncatula* snails. The prevalence of *Fasciola* sp. in the current research was more than the reported prevalence in animals with liver lesions in a slaughterhouse in Algeria (Ouchene-Khelifi et al., 2018). The prevalence rates of animals in the mentioned study were 26.7 ± 2.5%, 6.5 ± 0.4%, and 2.5 ± 0.2% in cattle, sheep, and goats, respectively. Therefore, the infestation level of ruminants at the slaughterhouses did not correspond to the infestation level of snails with *Fasciola* sp. in the pasture.

In Constantine, the molecular prevalence rate of *Fasciola* sp. in snails was 25%, compared to 26.2% in Tunisia (Hammami and Ayadi, 2000; Hammami et al., 2007) and 26.6% in Poland (Kozak and Wedrychowicz, 2010). However, in El Tarf, the prevalence was very high (42.85%). This could be explained firstly by the humid climate of this region which eases the development of snails and facilitates their contamination by *F. Hepatica*. Secondly, the size of the snails in this area which is the largest in the region of El Tarf supports the high prevalence in this region (Rondelaud et al, 2014).

In the present investigation, the survival rates of snails in the three tanks for 6 weeks were relatively high, compared to the results of Righi et al. (2016) in Algeria (average rates vary from 28 to 30%). The differences could be explained by the fact that the collected snails can be contaminated by larvae of some flies that can kill them (Muniz-Pareja and Iturbe-Espinoza, 2018). Moreover, the findings indicated that the survival rate in tank 3 (adult snails) was the lowest (40%), which was in agreement with some other studies where the mortality rate was higher in adult snails than in younger ones (Rondelaud et al., 2009; Righi et al., 2016).

The growth rate of snails depends largely on the environment in which they live, meaning that they grow better in calcareous or siliceous soil (Vignoles et al., 2010; Rondelaud et al, 2014; Vignoles et al., 2017). However, in experimental conditions like the present study, the growth rate was largely dependent on the initial size of the snail. Thus, the smallest snails (3 to 4 mm), recorded the greatest growth (3mm). On the other hand, the larger snails (7-8 mm) recorded a low growth rate (1.1 mm). It seems clear that the smaller snails are growing faster.

The largest number of laying was recorded in tank 3 (136), while in tank 1, only 29 were recorded. This was due to the fact that the snails in tank 3 (over 7 mm) were sexually mature and laid more eggs, which naturally affected all other fertility parameters.

CONCLUSION

The use of multiplex PCR clearly indicated a relatively high level of infestation of the snails by *Fasciola* sp. The survival rate of the snails under the experimental conditions varied between 40 and 50%. The growth rate of the snail

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depended on the initial size of the snail. Snails larger than 7 mm seemed to be the most mature ones and had the highest lay rate. Further studies with a higher number of snails and in other regions of Algeria could be carried out in order to have more information and to investigate other snails that could be infested by *Fasciola* sp.

DECLARATIONS

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Authors' contribution

Fayçal Aimeur, Meriem Mekroud, and Amal Titi conceived and designed the research. Nassim Ouchene and Abdeslam Mekroud analyzed the data, Nadjet Amina Ouchene-Khelifi and Abdeslam Mekroud wrote the manuscript. All authors checked and approved the final version of the manuscript.

Competing interests

There is no conflict of interest.

Consent to publish

All authors approved the final version and agreed to publish the article in the present journal.

Ethical consideration

The authors checked for ethical issues including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

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