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SUSCEPTIBILITY OF SOME FRESH WATER SNAILS OF PORTUGAL AND SPAIN TO Fasciola hepatica (1)

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INTRODUCTION

Fascioliasis poses a sanitary and economic problem of great significance to Portugal, as evidenced not only by the high animal infection rates, but also the constant increase of human cases (Xavier & coll., 1965).

In fact, after the 25 human cases described, ever since the first one that occurred in 1948 (Fonseca, F. & Azevedo, F.) there have been, at least, 15 more, in last two years that we are aware of. The fact that both the previous cases and the recent ones have been described without systematic research, as well as the wide area of distribution of the disease in animals in the several districts of our Country, and the death of sheep and bovines in 1964 (Xavier, loc. cit.) and again in 1966 and 1967 in several northern regions, lead us to conclude that fasciliasis is much more serious, as far as our Country is concerned, than one would at first believe. Accordingly, we must try to identify with accuracy the vector snails and their area of distribution, in order to do ecologic research and to clarify the host-parasite relationship which is the base of the epi-

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demiology and epizootiology and subsequent control and prophylaxis of this parasitosis.

It is a well know fact that *Lymnaea truncatula* is the natural intermediate host of *Fasciola hepatica*, and the Iberian Peninsula is no exception to this (XAVIER, M. L., loc. cit.; GONZALEZ CASTRO, J., 1947).

Other *Lymnaeidae* may act as relative hosts: *L stagnalis, L. peregra, L. glabra* and *L. palustris* (KENDALL, S. B., 1965; BERGHEN, P., 1964); the first two are the most susceptible. One may accordingly assume that only *Lymnaea* species are able to act as intermediate hosts, with the absolute exception of *L. auricularia*, related with the *F. gigantica* vetors (KENDALL, S. B. loc. cit.).

According to BERGHEN (loc. cit.) and XAVIER (loc. cit.), and according to the experience of most authors doing field work in many fascioliasis areas malacological research often confirm the intense occurence of other snails (*Physsa* and other *Lymnaeidae)* whereas *L. truncatula* is very seldom, or even not at all, found in these areas; this leads us to suspect that there may other snails acting as intermadiate hosts.

Among the genus *Physa, P. acuta* has the widest distribution, due to its great reproductive capabilities and resistance to poor nutrition and other adverse conditions.

Some *Physa* species are considered potential hosts, as is the case with *P. cubensis* (VIGUERAS, J. & MORENO, B. A., 1935) and *P. fontinalis* (BOYD, M. F., 1920), quoted by BRUMPT.

These two reasons, i. e., the dense distribution of *Physa* in fascioliasis areas and the fact some of the species act as hosts, make it imperative to ascertain whether they are not *F. hepatica* vectors. Accordingly, one of the objetives of the present work is to assess the susceptibility of *P. acuta* and other snails to the *F. hepatica* under laboratory conditions and using *L. trucatula* as control. This implies the existence of a previous culture of both snails, and technical and experimental research on the biological cycle of *F.hepatica in L. truncatula,* which is being maintained in our laboratory since 1964 (XAVIER, M. L. & Coll., 1966).

Since the role of *L. truncatula* as a vector of *F. hepatica* in Spain has not been completely confirmed, we have also exposed this species, as well as *P. acuta, L. peregra* and *L. stagnalis* from Salamanca.

MATERIAL AND METHODS

Both the Portuguese and Spanish populations of *Physa* and *Lymnaea* used in our experiments were maintained in cultures according to the method employed by Ollerenshaw, C. B. in the Central Veterinary Laboratory, Weybridge, England, for *L. truncatula.* The culture is done in plastic containers on whose earth-covered bottom a culture of the alga *Oscillatoria formoso* Bory is established. The culture of *Oscillatoria* is optimum for all snails, specially *Physa,* because they grow rapidly, attain sexual maturity much earlier and reproduce intensely, as was reported for other species (XAVIER , M. L. & Coll., 1966).

The miracidia were obtained from infected sheep liver blade, according to the technique used at Weybridge (Rowcliffe & Ollerenshaw, 1960)

Exposure is achieved by placing each snail in one haemolysis tube with some 2 ml of water and by keeping the snail in contact with the miracidia for 6 hours, under natural or artificial lighting. Mass exposure was also done: aech snail lot was placed on a new *Oscillatoria* plate and miracidia were introduced in the plate itself.

The penetration of miracidia and their resulting development in the tissues of the snails were assessed via series of histological sections, 8 microns thick, of the head and foot regions of *L. truncatula* and *P. acuta* specimens, taken 24, 72 and 96 hours after a massive exposure of 500 miracidia per snail.

Sections were achieved via inclusion in paraffin and dyed with hematoxilin-eosin. Other snails, intended for the observation of both sporocysts and rediae and cercariae, were dissected while still living, after being previously anesthetized, respectively 15 and 30 days after the exposure to infection.

After 30 days the remaining snails were exposed to cercaria shedding conditions in an ice bath at 8-10°C.

In some lots used for cercaria shedding we did not wait for spontaneous shedding; instead, we forced it by rupturing the first whorl, as advised by Berghen (loc. cit., 1964), since this facilitates cercaria shedding which was explosive.

The snail that did not shed were dissected as above in orden to observe larval forms.

EXPERIMENTS

Group I

1. *Exposure of average-sized P. acuta and L. truncatula*

We collected a total of 130 *Physa* with diameters ranging from 4 to 6 mm, descended from a population of Alcácer do Sal (Alentejo, South of Portugal), maintained in the Laboratory since 1964; 110 snails were exposed to infection with 50 miracidia per snail, and 20 were used as control.

The experiment was conducted as shown in Table I:

Lot FS_1 Composed of 50 specimens, of which 25 were kept at 30°C and 25 at room temperature. Both sets intended for dissection 15 and 30 days after exposure in order to observe sporocysts and rediae and cercariae, respectively.

Lot FS_2 Composed of 40 specimens, 20 of which were kept at room temperature and 20 at a constant 30°C. Both were intended for spontaneous shedding via an ice bath at 8-10°C. after 30 days of exposure to infection.

Lot FS_3 Composed of 20 snails, kept at room temperature and intended for forced shedding by rupture of the first whorl. (Berghn's method).

Lot FS_4 Control lot: 20 snails 10 kept at room temperatura and 10 at 30°C.

Simultaneously, 80 *L. truncatula* specimens were taken. They were of average size, came from a population of Vila do Conde (North of Portugal), and maintained in the laboratory since 1965. Forty were exposed to infection with 15 miracidia per snail, and 10 were used as longevity controls. This lot as a whole acted as control for the infective capabilities of the miracidia employed and, consequently, for the reproduction of the life-cycle of *F. hepatica.*

Infected snails were grouped in lots parallel (Table I) to the *Physa* lote, exposed to the same conditions and for similar purposes:

Lot LP_1 20 specimens, 10 for dissection after 15 days of exposure, and 10 for dissection after 30 days, all were kept at room temperature.

Lot. LP_2 40 specimens for spontaneous shedding after 30 days, 20 were kept at room temperature and 20 at 30°C.

Lot LP_3 20 specimens for forced shedding via rupture of the shell's first whorl.

Lot LP_4 20 longevity controls, 10 at room temperature and 10 at 30°C.

Results:

Tests ended 40 days after exposure; mortality rate was 14.5 % in the *Physa* exposed to infection, and 0 % in the controls.

During the dissection after the first 15 days of the *Physa* and *Lymnae* of lots FS_1 and LP_1, respectively, no sporocysts were reported in any of the 18 *Physa* dissected; among the 6 *Lymnaea,* several sporocysts were reported in 4. In the dissection after 30 days, no larval forms were reported in any of the 19 *Physa* dissected; among 8 *Lymnaea,* 5 proved to have mature rediae and cercariae that encysted in the glass, resulting in metacercariae and immature cercariae that did not encyst.

Spontaneous shedding was not obtained in *Physa,* whereas a percentage of 78.9 was noticed in *Lymnaea.*

The snails that did not shed after 40 days were dissected and larval stages were found in several *Lymnaea* and none in *Physa.* The same was true for the groups intended for forced shedding; it was explosive in *Lymnaea* and did not occur in *Physa.*

We also exerted daily control of the number of egg-masses of each of the snail species. Table II shows the distribution of the number of egg-masses per snail, both before exposure and from the 1st to 15th day and from the 16th to the 30th day after exposure: in *L. truncatula,* coinciding with the migration of the larval forms to the hepatopancreas, we observed that egg-laying began to decrease after the 15th day of exposure and became nil in all lots at the end of the test. This was not true for *Physa,* thus proving that the attempt at infection had failed.

Conclusions:

The experimental infection of the average-sized *P. acuta* (4 to 6 mm diameter) was not achived, but an infection of 90 % was reported in *L. truncatula* by final dissection of survivors after 40th day of exposure. The 14.5 % mortality reported in *Physa* was due to exposure conditions as it was 0 % in the *Physa* controls. In *L. truncatula,* mortality until the 40th day reached 30 % and was due to infection, since controls had a 0 % mortality.

2. *Hystological comparison of miracidia penetration*

It was possible to report aporacysts in the head and foot of 4 of the 6 *L. truncatula* specimens exposed, as may be seen in fig. 1 for one of them; however, in the 6 *Physa* specimens exposed to the same conditions of infection it was not even possible to observe the penetration of miracidia.

Group II

These tests, conducted with *P. acuta* only, were intended to determine whether the number of miracidia, age, temperature and origin of the snail population influenced susceptibility to infection.

The sequence of the experiment is shown in Table III.

In the first group of tests, some specimens from Alcácer do Sal, were exposed to infection with 2 to 8 miracidia per snail and then divided into two lots:

Lot AS_1 Composed of 30 snails kept at room temperature.

Lot AS_2 Composed of 30 snails kept at 30°C.

Lot TAS_1 and TAS_2 were the respective controls.

The same technique was employed with another group of specimens from the same area, the only difference being thet both snails exposed to infection and the controls were 6 days old.

Still the same method was employed with specimens from Vila do Conde (Portugal) and Salamanca (Spain), 2 and 6 days old.

The same procedure was followed for the second, third and fourth groups of tests, except that specimens of the three populations were exposed to a different number of miracidia-respectively 15 to 30, 40 to 50, and mass exposure, as described before.

Results:

The results obtained are shown in Table IV:

A total of 1,540 snails was exposed, 790 of which were kept at 30°C and 820 at room temperature. Mortality due to the experimental conditions was 13.9 % in the former case and 20.3 % in the latter, while controls' mortality was 6.4 % and 7.5 % respectively.

Medium initiation of egg-laying, determined only in the first group (snails from Alcácer do Sal, 2 days old) took place ofter 22.8 days of exposure, with a medium age of 27.2 days in exposed snails at room temperature, and after 21.6 days of exposure, with a medium age of 26.6 days in the snails kept at 30°C.

Medium number of egg-masses of the total exposed snails at room temperature was 5,739, while it was 1,913 for the control snails, which is equivalent to 6.9 egg-masses per each exposed snail and 6.8 per control snail. Snails kept at 30°C had a total of 6,623 egg-masses, while controls had 2,306 i. e., 8,3 and 9.2 egg-masses per snail, respectively. One may conclude, then, that the temperature of 30°C favours oviposition but does not affect susceptibility to infection.

The experiment ended after 40 days and, as the snails did not shed cercariae, the 530 snails of the 4 groups were dissected and no larval forms of *F. hepatica* were found in any of them.

Group III

Though no *L. stagnalis* specimens have been found in the fasciolíasis areas of Portugal we have observed, since this species may be found in association with *L. truncatula* in Spain and due, to the previously stated reasons, we decided to expose it to experimental infection together with *L. peregra* and *L. palustris*, since the former is very common and the latter very rare in the mentioned areas.

L. truncatula from Salamanca, was also exposed, with *L. truncatula* from the Oporto District (north of Portugal), being the last one as control of all experiments.

We used 10-20 miracidia for *L. truncatula*, and 40-50 for the other *Lymnaeidae* under several experimental conditions as are shown in Table V. Hystological observations of this species were also done with the purpose of observing larval stages of *F. hepatica*.

Results:

From a total of 320 *L. stagnalis* specimens (Table V) exposed to infection, 55 (17.1 %) died, whereas there was a 10 % mortality in control snails. However, this species proved to be impervious to infection under any conditions.

The same was noticed with both the Portuguese and Spanish *L. peregra* with mortality rate of 17.3 and 18.9 %, respectively, while control snails had 10.0 % mortality for the Portuguese species and 13.3 % for the Spanish ones.

In the three snail species exposed to infection, mortality was generaly higher than in controls, which must be due to the conditions to which the snails are subjected during exposure.

The histological observation of these species revealed no sporocysts or other larval forms; however, the same was not true for the specimens of the Spanish population of *L. truncatula* (fig. 2) nor for the Protuguese specimens of the same species and of *L. palustris* (fig. 3).

Infection rates were respectively 52.2 %, 47.9 % and 10 %. In any these species, mortality rate was higher than in the control species (Table V), a fact that may be atributed not only to exposure conditions but to infection itself.

CONCLUSIONS

The fact that *P. acuta* and *L. peregra*, the snails most densely distributed in the fascioliasis areas of Portugal, have showed no susceptibility to *F. hepatica*, the fact that only the uncommon *L. palustris* has behaved as a relative vector and the fact that *L. stagnalis* has not even been reported yet in these areas, lead us to conclude that fascioliasis in Portugal is exclusively dependent on *L. truncatula*.

This led to a more detailed investigation on the species geografic distribution which enabled us to conclude, in a work still under progress, that this species is much more common in Portugal than was previously thought. Thus, for instance, in 1965, only 10 breeding-places of *L. truncatula* had been reported in Matosinhos country (Oporto District), and presently there already 150; the same is true for the other counties of the district: up to now, we have a total of 553 breeding-places.

Specimens of *L. truncatula* were also found by us in several areas of Alentejo and Algarve Provinces, though the species is more common in the north of the Country.

The initial lack of overlap in the occurrence of *L. truncatula* in fascioliasis areas (Xavier & Coll., 1965) should be atributed to our initial difficulties not only in locating the minute-size and amphibious snail in its different habitats, but also in distinguishing these same habitats types.

This is particulary true for the Province of Alentejo (southern Portugal), whose general caracteristics —climate and others— do not appear to be favourable to the evolution of fascioliasis, which, however, occur there in considerables rates, though in restricted areas, i. e., only wherever the microclimate is favourable to the development of *L. truncatula*.

In Spain, similary to what occurs in Portugal, *L. truncatula* is the main vector of *F. hepatica*, since *L. peregra*, *L. stagnalis* and *P. acuta* proved to be impervious to experimental infection.

As *L. stagnalis* sometimes occurs in association with *L. truncatula* in Spain, contrarily to what happens in Portugal, and as it is considered in some countries as one the most adequate relative hosts of *F. hepatica* (Kendall, loc. cit.; Berghen, loc. cit.), it becomes necessary to ascertain with accuracy the role of this species as a host. We were unable to do so because we have only one single snail population and it is a known fact that may be different susceptibilities among different populations of the same species.

RESUMEN

En las investigaciones malacológicas de campo, se comprueba la rareza de *L. truncatula* mientras que aparecen abundantemente *P. acuta* y *L. paregra*. Como quiera que estos dos últimos moluscos actúan como vectores relativos de Fasciola en otros países, se hace necesario conocer si son o no hospedadores intermediarios del tremátodo en la Península Ibérica.

De los tres grupos de experiencias realizadas se deduce que la fasciolosis depende, en la Península Ibérica, principalmente de *L. truncatula*. Ejemplares portugueses de *L. palustris* actuaron también como vectores relativos, con tasas de infestación del 10 %. No pudo ser demostrado ningún tipo de infestación experimental en *L. stagnalis*, *P. acuta* y *L. peregra* procedentes de ambos países.

RESUME

Dans les recherches malacologiques effectuées à la campagne on observe la rareté du *L. truncatula*, alors que le *P. acuta* et le *L. paregra* se trouvent en grand nombre. Etant donné que ces deux derniers mollusques agissent comme des vecteurs relatifs de Fasciola dans d'autres pays, il faut savoir s'ils sont ou non des hôtes intermédiaires du trématode dans la Péninsule Ibérique.

Des trois groupes d'expériments effectués on déduit que la fasciolose, dans la Péninsule Ibérique, dépend principalement du *L. truncatula*. Des exemplaires portugais de *L. palustris* agirent aussi comme des vecteurs relatifs, avec un taux de 10 % d'infestation. On ne trouva aucun type d'infestation expérimentale chez les *L. stagnalis*, *P. acuta* et *L. peregra* provenant de ces deux pays.

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SUMMARY

Malacological Research has often confirmed that *L. truncatula* is only seldom, or even not at all found in fascioliasis areas, while *P. acuta* and *L. peregra* show a wide dense distribution; on the other hand, these snails may act as relative vectors in other Countries. The question, therefore, arises of the whether or not they are vectors for *F. hepatica* in the Iberian Peninsula.

From the experiments conducted it may be concluded that fascioliasis in the Iberian Peninsula depende mainly on *L. truncatula* since, in Spain, none of the other snails tested was shown to be a vector, and in Portugal only *L. palustris* could be shown to act as a relative vector. Infection rates were 52.5 %, 47.9 % and 10.0 %, respectively.

This led to a more detailed investigation on the species geographical distribution on Portugal which enabled the authors to conclude, that *L. truncatula* is more common in this Country than was previously thought. The initial lack of overlap found in the occurrence of *L. truncatula* in fascioliasis areas should be atributed to initial difficulties not

only in locating the minute-size and amphibious snails in their different habitats, but also in distinguisly the same habitats types.

From the three groups of experiments conducted it may be concluded that fascioliasis in the Iberian Peninsula depends mainly on *L. truncatula*. *L. stagnalis*, *P. acuta* and *L. peregra* from both countries showed resistance to infection.

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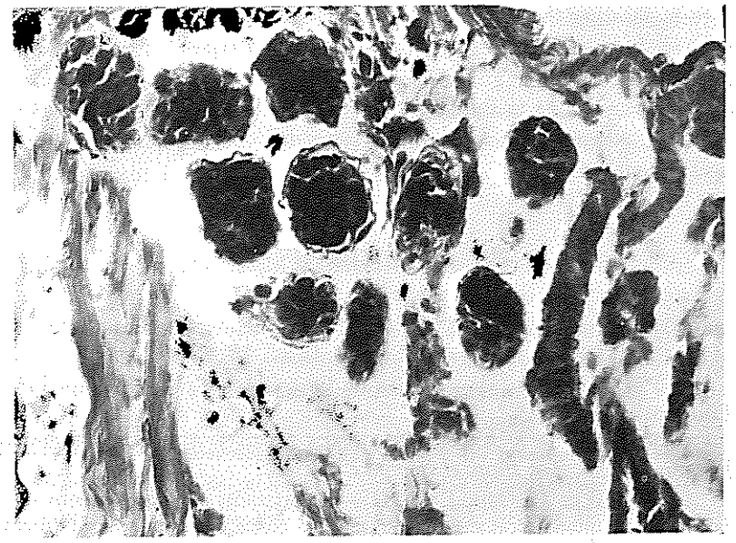

Fig. 1.—Sporocysts in evolution in a specimen of *L. truncatula* from Vila do Conde, Portuga, 72 hours after exposure to infection. (350 ×).

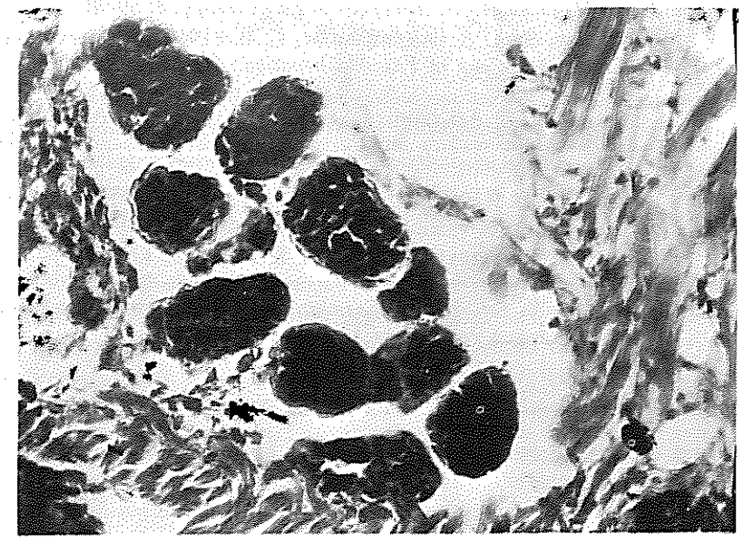

Fig. 2.—Sporocysts in evolution in a specimen of the Portuguese *L. palustris*, 72 hours after exposure to infection. (350 ×).

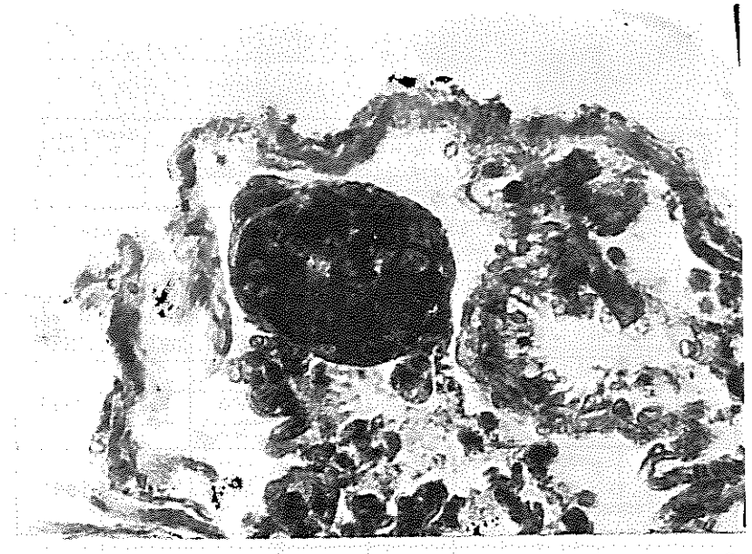

Fig. 3.—Sporocysts in evolution in a specimen of *L. truncatula* from Salamanca (Spain), 96 hours after exposure to infection. (365 ×).

Species of snails	Snails exposed to the laboratory infection										
	Conditions of experiment	Snail lots number	Number of snails	Number of dead snails	Percent of mortality	Number of snails with sporocysts	Number of snails with rediae and cercariae	Number of snails with spontaneous cercariae	Number of snails infected	Rate of infection (%)
P. acuta	Dissection at 15 days and 30 days	FS_1	50	12	24.0	—	—		—	
	Spontaneous shedding	FS_2	40	2	5.0			—	—	
	Berghen's method	FS_3	20	2	10.0		—		—	
	TOTAL		110	16	14.5					
L. truncatula	Dissection at 15 days and 30 days	LP_1	20	6	30.0	4	5		9	
	Spontaneous shedding	LP_2	40	10	25.0			20	20	66.6
	Berghen's method	LP_3	20	8	40.0			9	9	75.0
	TOTAL		80	24	30.0			29	38	67.8

Table 1.—Results obtained in specimens of *P. acuta* and *L. truncatula* of Portuguese origin, when exposed to infection with *F. hepatica* miracidia.

		Snails at 30°C			Snails at room temperature		
	Experiment groups	Number of snails	Percent of snails	Number of egg-masses	Number of snails	Percent of mortality	Number of egg-masses
Exposed snails	1st 2 - 8 miracidia	230	13.0	1990	210	23.8	1484
	2nd 15 - 30 miracidia	150	16.6	1203	150	24.0	991
	3rd 40 - 50 miracidia	150	13.3	1413	180	22.8	1300
	4th mass of miracidia	260	13.5	2017	280	14.3	1964
	Total number	790	13.9	6623	820	20.3	5739
Control snails	Total number	250	6.4	2306	280	7.5	1913

Table II.—Egg-laying rates of Portuguese specimens of *P. acuta* and *L. truncatula* when exposed in the Laboratory to *F. hepatica* miracidia and subjected to different temperatures.

Snails exposed to the laboratory infection	Experiments groups	Number of miracidia	Number of dead snails and number of egg-masses at 30°C						Number of dead snails and number of egg-masses at room temperature			
			Age	Origin	Snail lots number	Number of snails	Number of dead snails	Number of egg-masses	Snail lots number	Number of snails	Number of dead snails	Number of egg-masses
	1st	2 - 8	2 days	Alcácer do Sal	AS_2	40	5	350	AS_1	30	10	320
				Vila do Conde	V_2	40	5	480	V_1	20	5	201
				Salamanca	A_2	40	10	330	A_1	40	10	230
			6 days	A. do Sal	AS_{10}	40	6	324	AS_9	30	6	220
				V. do Conde	V_{10}	30	4	205	V_9	40	15	283
				Salamanca	A_{10}	40	1	301	A_9	40	5	220
				Total of each group		230	30	1990		210	50	1484
	2nd	15 - 30	2 days	A. do Sal	AS_4	20	4	200	AS_3	30	2	195
				V. do Conde	V_4	30	3	220	V_3	20	5	123
				Salamanca	A_4	30	4	201	A_3	20	5	298
			6 days	A. do Sal	AS_{12}	30	5	230	AS_{11}	20	4	101
				V. do Conde	V_{12}	20	6	190	V_{11}	30	10	105
				Salamanca	A_{12}	20	4	162	A_{11}	30	10	209
				Total of each group		150	25	1203		150	36	991
	3rd	40 - 50	2 days	A. do Sal	AS_6	40	5	350	AS_5	20	5	250
				V. do Conde	V_6	20	0	210	V_5	30	2	275
				Salamanca	A_6	20	1	193	A_5	30	10	101
			6 days	A. do Sal	AS_{14}	20	4	200	AS_{12}	20	3	205
				V. do Conde	V_{14}	30	3	245	V_{13}	40	11	244
				Salamanca	A_{14}	20	7	215	A_{13}	40	10	225
				Total of each group		150	20	1413		180	41	1300
	4th	mass	2 days	A. do Sal	AS_7	50	10	400	AS_8	40	10	291
				V. do Conde	V_7	40	5	361	V_8	50	5	320
				Salamanca	A_7	50	5	413	A_8	40	4	305
			6 days	A. do Sal	AS_{15}	40	7	230	AS_{16}	50	3	410
				V. do Conde	V_{15}	40	3	302	V_{15}	50	7	323
				Salamanca	A_{15}	40	5	320	A_{16}	50	11	315
				Total of each group		260	35	2017		280	40	1964
Exposed snails				Total of all groups		790	110	6623		820	167	5739
Control snails				Total of all groups		250	16	2306		280	21	1913

Table III.—Study of the influence of the number of miracidia, age, origin of the snail populations and temperature on the susceptibility to Laboratory infection of *P. acuta* specimens.

Species of snails	Experimental conditions	Exposed snails		Control snails	
		From day 1 to day 15	From day 16 to day 30	From day 1 to day 15	From day 16 to day 30
L. truncatula	Room temperature	8.9	2.4	7.6	9.0
	at 30°C	4.5	3.5	14.5	8.0
P. acuta	Room temperature	6.4	8.5	14.0	11.0
	at 30°C	9.2	7.2	15.4	11.8

Table IV.—Joint results of the experimental exposure to infection of specimens of *P. acuta*, subjected to the conditions in Table III.

Species of snails and origin	Snails exposed to the laboratory infection										Control snails		
	Conditions of experiment	Snail lots number	Number of snails	Number of dead snails	Percent of mortality	Number of snails with sporocysts	Number of snails with rediae and cercariae	Number of snails with spontaneous cercariae	Number of snails infected	Rate of infection (%)	Number of snails	Number of dead snails	Percent of mortality
L. glabra (Salamanca)	Dissection at 15 days and 30 days	LP_1	150	20		—	—	—	—				
	Spontaneous shedding	LP_2	100	20		—	—	—	—				
	Berghen's method	LP_3	70	15		—	—	—	—				
	TOTAL		320	55	17.1						40	4	10.0
L. truncatula (Salamanca)	Dissection at 15 days and 30 days	TS_1	50	16		8	8		14				
	Spontaneous shedding	TS_2	70	21				32	32				
	Berghen's method	TS_3	40	10			13		13				
	TOTAL		160	47	29.3	8	21	32	59	52.2	30	2	6.6
L. truncatula (Oporto)	Dissection at 15 days and 30 days	TP_1	20	10		2	2		4				
	Spontaneous shedding	TP_2	60	10				24	24				
	Berghen's method	TP_3	20	7			7		7				
	TOTAL		100	27	27.0	2	9	24	35	47.9	20	1	
L. peregra (Oporto)	Dissection at 15 days and 30 days	PP_1	70	10		—	—	—	—				
	Spontaneous shedding	PP_2	80	15		—	—	—	—				
	Berghen's method	PP_3	40	8		—	—	—	—				
	TOTAL		190	33	17.3						30	3	10.0
L. peregra (Salamanca)	Dissection at 15 days and 30 days	PS_1	150	20		—	—	—	—				
	Spontaneous shedding	PS_2	100	25		—	—	—	—				
	Berghen's method	PS_3	120	15		—	—	—	—				
	TOTAL		370	70	18.8						30	4	13.3
L. palustris (Oporto)	Dissection at 15 days and 30 days	LP_1	70	15		3	2		5				
	Spontaneous shedding	LP_2	70	16				5	5				
	Berghen's method	LP_3	80	24			7		7				
	TOTAL		220	55	26.8	3	9	5	17	10.5	20	2	10.0

Table V.—Susceptibility of several fresh water snails from Portugal and Spain to *F. hepatica*.