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Developmental differences in the uterus of *Fasciola hepatica* between livestock liver fluke populations from Bolivian highlands and European lowlands

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Abstract A morphometric study of the uterus area (UA) of *Fasciola hepatica* adults was carried out with the aid of a computer linked to a stereomicroscopic 3CCD colour video camera using image analysis software. The UA of adult liver flukes found in naturally infected sheep, cattle and pig from the endemic human fascioliasis zone of the northern Bolivian Altiplano highlands was compared with that of flukes found infecting sheep and cattle from Valencia, Spain and cattle from Corsica, France (collectively, European lowlands). Liver fluke UA was examined using an allometric model. A comparison of the allometry of the liver fluke UA in different host species from Bolivia revealed no significant differences. Similarly, no statistically significant differences were found between UA of Valencian and Corsican populations. However, the Bolivian sheep and cattle liver fluke populations proved to have a UA smaller than that of the European populations. These results indicate differences between the liver fluke population of highlands and lowlands, regarding UA. This paper discusses the possible relationship between the characteristic of having a reduced uterine development and the liver fluke adult stage adapting to host populations living at very high altitudes.

Introduction

Fascioliasis due to the digenean species *Fasciola hepatica* has recently proved to be an important health problem (Chen and Mott 1990; Mas-Coma et al. 1999a, 2000), with human cases being reported in countries on all five continents (Esteban et al. 1998) and human endemic areas ranging from hypo- to hyperendemic (Mas-Coma et al. 1999b). A singular epidemiological characteristic of human fascioliasis is the link between hyperendemic areas and very high altitude regions, at least in South America (Mas-Coma et al. 1999a, b).

The highest prevalences and intensities of human fascioliasis known are found in the northern Bolivian Altiplano, at an altitude of 3,800–4,100 m (Hillyer et al. 1992; Mas-Coma et al. 1995; Bjorland et al. 1995; Esteban et al. 1997a, b, 1999; O'Neill et al. 1998; Mas-Coma et al. 1999c), where climatic conditions are extreme (Fuentes et al. 1999). Different studies have proven that *Lymnaea truncatula* is the only intermediate snail host species in this endemic region (Oviedo et al. 1995; Bargues and Mas-Coma 1997; Bargues et al. 1997; Jabbour-Zahab et al. 1997; Samadi et al. 1999). Results suggest a recent introduction into Bolivia of both snail intermediate host and parasite from Iberian populations (Valero et al. 1999); and multidisciplinary studies are ascertaining how the liver fluke and its lymnaeid hosts adapt to the extreme environmental conditions at high altitude and succeed in producing high infection rates in both humans and animals (Mas-Coma et al. 1999c).

According to livestock and sylvatic animal populations inhabiting this endemic region and to infection prevalences and intensities, sheep and cattle may be considered the main reservoir host species, with pigs and donkeys playing a secondary role (Ueno and Morales 1973; Ueno et al. 1975; Mas-Coma et al. 1995, 1997; Hillyer et al. 1996; Buchon et al. 1997; Fuentes et al. 1997; Grock et al. 1998). Valero and Mas-Coma (2000) have demonstrated that the viability and infectivity of metacercariae obtained experimentally from eggs shed by sheep, cattle, pigs and donkeys are similar.

Among the research activities carried out to characterise the transmission pattern of the liver fluke in the northern Bolivian Altiplano, special attention has been focused on adult development in the definitive host.

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In the high altitude environment, oxygen and air density decrease, temperature and humidity are low and there is an increase in radioactivity. These environmental factors exert an influence on the vertebrates; and those born and living at high altitude show different morphological and physiological characteristics to those inhabiting low altitudes. These changes may influence the development of a liver fluke such as *F. hepatica*, mainly because of its tissue migration and haematophagous diet (Dawes and Hughes 1964; Boray 1969). To ascertain whether geographic isolation and adaptation to high altitude have given rise to a parasite morph divergence (as detected in Altiplanic lymnaeid snails – see Oviedo et al. 1995), different morpho-anatomical aspects of liver fluke adults additional to the characteristics already analysed by Valero et al. (1999) are currently being studied. The aim of the present paper is to analyse the results obtained in the studies on uterus development.

Our study concerns uterus size versus body surface in *F. hepatica* adult populations. In *F. hepatica*, Neuhaus (1978) has already showed that the length of the uterus in *F. hepatica* at different stages of development is correlated with the size of the body. In the present paper, a comparison is made with the equation described by Valero et al. (1991, 1996, 1999). To analyse the influence of host species on the development of the liver fluke uterus at high altitudes, Bolivian Altiplanic liver fluke populations from three different host species (sheep, cattle and pig) were studied. To analyse the influence of altitude on the development of the liver fluke uterus, populations of the parasite obtained from the same livestock species but born and living in highlands (Bolivian Altiplano) and lowlands (European Mediterranean area) were compared. Liver fluke populations from highland sheep versus lowland sheep and from highland cattle versus lowland cattle are compared separately. *F. hepatica* does not frequently infect pigs in Europe, so no comparison for highland pigs versus lowland pigs could be made.

Materials and methods

Parasites

All the liver fluke material was obtained from the bile ducts of naturally infected livestock. All the adults were gravid specimens.

In Bolivia, adult *Fasciola hepatica* worms were recovered in Batallas and El Alto slaughterhouses, from animals living in the endemic area of the Bolivian Altiplano region between Lake Titicaca and the valley of La Paz, at an altitude of 3,800–4,100 m (Departamento de La Paz). The worms analysed were: 118 adults from nine Merino sheep (6–21 each), 57 adults from six Charolesa cows (3–15 each) and 68 adults from six pigs (5–21 each).

In Europe, adult worms were recovered in Cullera and Massamagrell (Valencia, Spain) and Portovecchio (Corsica, France), from animals living in geographical areas close to sea level. The worms analysed from Spain were 63 adults from 11 Merino sheep (2–10 each) and 48 adults from six Charolesa cows (4–12 each). The worms analysed from Corsica were 63 adults from seven Corsica cows (5–10 each).

Adult worms were fixed in Bouin's solution between a slide and coverglass but without coverglass pressure, then stained with Grenacher's borax carmine and mounted in Canada balsam.

Measurement techniques and data analyses

The measurements included body area (BA) and uterus area (UA; Table 1). The measurements were made with the aid of a computer linked to a stereomicroscope 3CCD colour video camera (Sony DXC-930P) using image analysis software (Optimas 5, Optimas Corporation, Seattle).

Allometry

For an accurate morphometric comparison, account must be taken of the increases in the different biometric parameters which occur during digenean development within the definitive host according to growth laws (Dawes and Hughes 1964; Valero et al. 1996, 1998). If adult populations of different ages are studied, morphometric differences attributable to age can appear. When studying natural populations, only the allometric growth of a given biometric measurement can be calculated as a function of another biometric measurement (Valero et al. 1991, 1999). The classic allometric equation (for constant differential growth rates) described by Huxley (1972) is usually used.

To study the relationship between two morphometric variables (y_1 and y_2) in adult flukes, we employed the function proposed by Valero et al. (1996, 1999), which is an alternative allometric function for *F. hepatica* adults based on logistic growth laws (variable differential growth rates) versus time: $(y_{2m}–y_2)/y_2 = c[(y_{1m}–y_1)/y_1]^b$, where y_1 is BA, y_2 is UA, y_{1m} and y_{2m} are maximum values towards which BA and UA respectively tend, and b and c are constants (Table 2).

To calculate the y_m asymptotic values, we employed a procedure consisting of simultaneously testing successive values for y_{1m} and y_{2m} with the least squares residual (sse).

Statistical data

Data processing was carried out with Cricket and SPSS software (Macintosh). Adjusted non-lineal curves were tested using r^2 and sse. For the comparison of allometric curves, log e transformations were necessary: $t\text{BA} = \ln[(\text{BAmax}–\text{BA})/\text{BA}]$ and $t\text{UA} = \ln[(\text{UAmax}–\text{UA})/\text{UA}]$. Differences in allometric curves were sought by analysis of covariance (ANCOVA; one-way analysis of variance design with one covariant) using initial tBA as a covariant. The growth rates of adults from the two populations were compared using the same y_m (for the highest of the analysed populations). The effect-size measures were controlled by the Power and the ETA

Table 1 Comparative morphometric data for *Fasciola hepatica* adult populations from the Bolivian Altiplano, Valencia and Corsica in cattle, sheep and pig. All values are shown as range, with mean ± SE in parentheses. *BA* Body area, *UA* uterus area

	Sample size	BA (mm^2)	*UA* (mm^2)
Cattle Altiplano	57	19.06–196.35 (120.46 ± 35.43)	1.22–8.69 (4.26 ± 1.88)
Valencia	48	58.15–167.27 (113.80 ± 25.72)	1.75–11.85 (5.76 ± 2.16)
Corsica	63	48.99–246.97 (142.43 ± 42.62)	1.350–11.07 (6.05 ± 2.13)
Sheep Altiplano	118	39.17–207.39 (96.60 ± 39.24)	0.22–7.15 (3.08 ± 1.44)
Valencia	63	75.09–239.13 (140.36 ± 34.15)	0.87–13.65 (5.90 ± 2.24)
Pig Altiplano	68	45.37–182.03 (108.11 ± 29.92)	0.89–8.82 (3.87 ± 1.88)

Table 2 Allometric function $\{(y_{2m}-y_2)/y_2 = c[(y_{1m}-y_1)/y_1]^b$; see text$\}$ obtained from a logistic model with respect to time in adult worms of *F. hepatica* from the Bolivian Altiplano, Valencia and Corsica in cattle, sheep and pigs. *b, c* constants, r^2 correlation coefficient, *SE* standard error, *sse* least squares residual, y_{1m} maximum value of body area, y_{2m} maximum value of uterus area

	y_{1m}	y_{2m}	$b \pm$ SE	$c \pm$ SE	r^2	sse
Cattle Altiplano	245	8.70	1.033 ± 0.185	1.014 ± 0.091	0.45	112.03
Valencia	417	11.20	1.378 ± 0.158	0.322 ± 0.040	0.65	95.49
Corsica	410	11.85	1.644 ± 0.291	0.214 ± 0.063	0.44	125.99
Sheep Altiplano	327	7.60	1.130 ± 0.097	0.529 ± 0.052	0.60	94.31
Valencia	256	14.00	0.676 ± 0.113	1.612 ± 0.115	0.41	84.32
Pig Altiplano	525	8.90	1.720 ± 0.287	0.125 ± 0.048	0.43	136.05

statistics, used to describe the proportion of the total variability as "explained" by the grouping or factor variable (Norusis 1994).

Results

Analysis of the influence of the host species on liver fluke uterine development

Significant ANCOVA differences ($P < 0.05$) in *t*UA/*t*BA between Bolivia liver fluke populations from cattle, sheep and pig were not detected (Power = 0.77; ETA = 3.7%). Figure 1A shows the overlap of UA versus BA values in the liver flukes obtained from the three Bolivian host species.

The functional allometric shape of uterine development versus body size was not significantly different between the European populations. No significant ANCOVA differences ($P < 0.05$) were detected in *t*UA versus *t*BA between cattle liver fluke populations from Spain and Corsica (Power = 0.67; ETA = 5.2%).

Comparison of uterine development between Bolivian and European liver flukes

The functional allometric shape of uterine development versus body size was significantly different between Bolivian and European populations.

Significant ANCOVA differences ($P < 0.05$) were detected in *t*UA versus *t*BA between cattle liver fluke populations from Bolivia and Spain (Power = 0.97; ETA = 12.4%); and significant ANCOVA differences ($P < 0.05$) were also detected in *t*UA versus *t*BA between cattle liver fluke populations from Bolivia and Corsica (Power = 0.98; ETA = 12.4%). When the Bolivian liver fluke population from cattle had BA values similar to those of the European lowland populations, the Bolivian flukes showed a tendency toward a smaller UA (Fig. 1B).

Significant ANCOVA differences ($P < 0.05$) were detected in *t*UA versus *t*BA between sheep liver fluke populations from Bolivia and Spain (Power = 0.99; ETA = 12.4%). At similar BA values, the Bolivian liver fluke population from sheep tended to have a UA smaller than that of the populations from European lowlands (Fig. 1C).

Discussion

Several mammalian species may serve as definitive hosts for *Fasciola hepatica*, but there is considerable variation in susceptibility and induced pathology according to host species. Ross (1968) classified animal hosts of *F. hepatica* in three groups: animals with low, medium or high resistance. Sheep belong to the low resistance group, cattle are in the medium resistance group and pigs belongs to the high resistance group. Haroun and Hillyer (1986) reviewed our knowledge on how this resistance, to a greater or lesser extent, affects *F. hepatica* adults. The present study shows that uterine allometry of the *F. hepatica* adult with respect to BA follows a pattern which is independent from the host species. It is of great interest that it follows the same pattern in Bolivian pigs as it does in hosts considered normal, such as sheep or cattle, because in other geographical areas the pig shows substantial natural resistance to infection by *Fasciola* species (Ashizawa et al. 1966; Oshima et al. 1971; Shimizu et al. 1994), or is even considered to be non-viable as a host for *F. hepatica* (Polyakova-Kr'steva and Gorchilova 1972).

The environmental factors at high altitude influence the body of vertebrates. It has been known for many years that animals born and living at high altitude have morphological and physiological characteristics different than those animals at low altitude. Vertebrates inhabiting high altitude zones exhibit changes such as hypoxia (Levine et al. 1988), alterations in immune response (Sarybaeva et al. 1988), elevated haematocrit levels, differences in blood oxygen pressure and blood viscosity (Sakai et al. 1984) and elimination of dissolved gases, especially N_2, from the blood (Hirai et al. 1988). Studies on phenotype variability in helminth populations parasitising hosts living under the extreme environmental conditions of very high altitudes are unfortunately lacking. Concerning *F. hepatica*, Valero et al. (1999) demonstrated that only slight differences can be found in the allometry of body measurements (body length, body width, and perimeter versus BA or body length) between the *F. hepatica* adult from highland and lowland populations of Bolivian and Spanish sheep. The present study demonstrates both in sheep and in cattle that the Bolivian highland liver fluke populations show a smaller UA

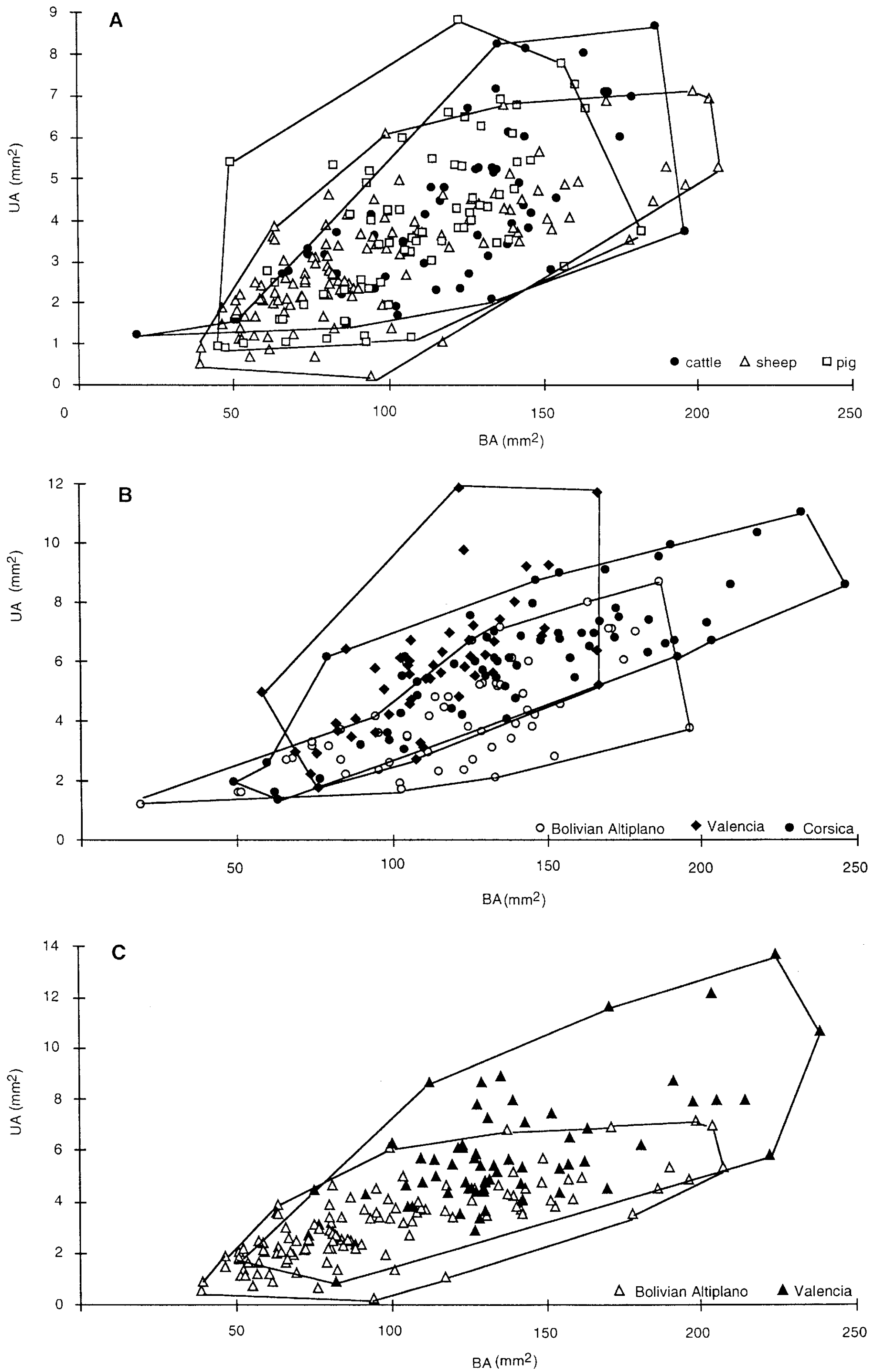

Fig. 1A–C Changes in uterus area (*UA*) as a function of the body area (*BA*) with the allometric model $(y_{2m}-y_2)/y_2 = c[(y_{1m}-y_1)/y_1]^b$ (see text) in adult worms of *Fasciola hepatica* from naturally infected livestock. Each point represents an adult individual. **A** Comparison of *F. hepatica* adult data for cattle (●), sheep (Δ) and pig (□) from the Bolivian Altiplano. **B** Comparison of *F. hepatica* adult data for cattle from the Bolivian Altiplano (○), Valencia (◆) and Corsica (●). **C** Comparison of *F. hepatica* adult data for sheep from the Bolivian Altiplano (Δ) and Valencia (▲)

development than the European lowland liver fluke populations.

Although it cannot be disregarded that this may be attributable, to some extent, to intraspecific variability not related to the influence of altitude, a divergence between highland and lowland populations in such a characteristic is difficult to understand, because any evolutionary advantages are not easily envisaged. It is known that oxygen is still required for egg production in *F. hepatica* (Mansour 1958; Bjorkman and Thorsell

1963; McGonigle and Dalton 1995). Thus, high altitude hypoxia could provide the origin for a reduced egg production by the flukes. Moreover, although the uterus in digeneans has traditionally not been considered as a storage organ but mainly as an organ adapted to the developmental time of the eggs (in fasciolids, eggs are laid unembryonated and the miracidium begins its development in eggs once in freshwater), our recent experimental studies with rats have demonstrated that there is a direct relation between *F. hepatica* uterus size and the number of eggs shed per gram of faeces (Valero et al., unpublished). In the northern Bolivian Altiplano, the climatic conditions, freshwater characteristics and lymnaeid ecology in this southern hemisphere latitude enable fascioliasis transmission to take place throughout the year (Fuentes et al. 1999; Mas-Coma et al. 1999c), so that egg storage a priori is not as essential as in the northern hemisphere latitudes, where fascioliasis transmission is typically seasonal. Further research is presently being carried out to ascertain whether the morphometric differences detected in the uterus of Bolivian *F. hepatica* adults are associated with biological differences.

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