Comparison of adult liver flukes from highland and lowland populations of Bolivian and Spanish sheep

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Abstract

A morphological study of adult liver flukes and eggs from sheep in a human fascioliasis endemic zone in the Northern Bolivian Altiplano showed that they belong to the species *Fasciola hepatica*. An exhaustive morphometric comparison with a *F. hepatica* population from Spanish sheep was made using image analysis and an allometric model: $(y_{2m}-y_2)/y_2=c[(y_{1m}-y_1)/y_1]^b$, where y_1 = body surface or body length, y_2 = one of the measurements analysed, y_{1m}, y_{2m} = maximum values towards which y_1 and y_2 respectively tend, and c, b = constants. Only slight allometric differences in worms were observed despite the geographic distance between both Spanish and Bolivian sheep populations and the very high altitude of the Bolivian Altiplano.

Introduction

Fascioliasis is an endemic disease in the Northern Bolivian Altiplano, a region located at a very high altitude (3800–4100 m) between Lake Titicaca and the valley of the city of La Paz. In this region, human prevalences and intensities are the highest known worldwide (Hillyer *et al*., 1992; Mas-Coma *et al*., 1995; Bjorland *et al*., 1995; Esteban *et al*., 1997a,b; Hillyer & Apt, 1997). Altiplanic livestock also present high indices of liver fluke infection (Ueno *et al*., 1975; Mas-Coma *et al*., 1995; Hillyer *et al*., 1996). Although always referred to as *Fasciola hepatica* (Linnaeus, 1758) (Trematoda: Fasciolidae), the identification of the parasite in this region has never been the subject of specific research. Altiplanic liver fluke species identification is important because of three aspects: (i) in other parts of the world *F. hepatica* does not reach such altitudes, being a parasite typical of low altitude zones (only up to around 2000 m altitude) as reviewed by Oviedo *et al*. (1995); (ii) the presence of both liver fluke species have been reported in the Americas: *F. hepatica* in North, Central and South America (Boray, 1969, 1982) and *F. gigantica* Cobbold, 1855 in Texas and Florida (Price, 1953); and (iii) *F. gigantica* shows a more tropical distribution than *F. hepatica* (Boray, 1982).

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 sheep, and those born and living at high altitude show different morphological and physiological characteristics to those inhabiting low altitudes (Jackson *et al*., 1987; Frisancho & Frisancho, 1992). Unfortunately, little is known about the effects of very high altitude upon parasite species.

The aims of the present paper include: (i) the specific identification of the liver fluke inhabiting the Northern Bolivian Altiplano; and (ii) the morphometric characterization of the adult stage of the Altiplanic liver fluke population. For these purposes, adult flukes found in naturally-infected Altiplanic sheep were studied. Although on the Bolivian Altiplano the fluke is known to infect both sheep and cattle, only parasites from sheep were analysed, taking into account that sheep are considered the typical host species for *F. hepatica* worldwide. Moreover, there are several studies which prove that sheep inhabiting high altitude zones exhibit several changes, such as hypoxia (Levine *et al*., 1988), alterations in immune response (Sarybaeva *et al*., 1988), elevated haematocrit levels, differences in blood oxygen pressure

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values and blood viscosity (Sakai *et al*., 1984) and elimination of dissolved gases, especially N_2, from the blood (Hirai *et al*., 1988). These changes may influence the development of a liver fluke such as *F. hepatica*, mainly because of its tissue migration and haematophagous diet (Dawes & Hughes, 1964; Boray, 1969). To ascertain whether geographic isolation and adaptation to high altitudes have given rise to a parasite morph divergence (as detected in Altiplanic lymnaeid snails – see Oviedo *et al*., 1995), a detailed morphometric comparison is made with adult liver flukes and eggs found in naturally-infected sheep from a low altitude area in Spain.

For an accurate morphometric comparison, increases in the different biometric parameters which occur during digenean development within the definitive host according to growth laws (Dawes & Hughes, 1964; Valero *et al*., 1996) must be taken into account. 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 as a function of another biometric measurement may be calculated (Valero *et al*., 1991). The classical allometry equation (constant differential growth rates) described by Huxley (1972) is usually used. Valero *et al*. (1996) proposed an alternative allometric function for adults of *F. hepatica* based on logistic growth laws (variable differential growth rates) versus time. In the present study a comparison is made of both equations between Bolivian and Spanish fluke populations.

Materials and methods

Parasites

All liver fluke material was obtained from the bile ducts of naturally infected Merino sheep (*Ovis aries*). In Bolivia, 112 adult worms (range of 6–21) were recovered from eight sheep in the area around the town of Batallas (provincia de los Andes, Departamento de la Paz), on the Bolivian Altiplano region between Lake Titicaca and the valley of the city of la Paz, at an altitude of 3800–4100 m. In Spain, 53 adults (range of 2–10) were recovered from 10 sheep in the towns of Cullera and Masamagrell, Valencia province, on the Mediterranean coast. These two populations were selected because they offered the largest worm variability (different stages of maturity, body size and gravid uteri). In addition, 70 eggs were obtained from filtering the gallbladder of some Bolivian sheep, whereas in Spain, 90 eggs were dissected out of the terminal part of the uteri of adult worms.

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

Measurement techniques and data analyses

All standardized measurements of adults were made according to methods proposed by Mas-Coma *et al*. (1984) for brachylaimid trematodes but modified by Valero *et al*. (1996) for the Fasciolidae. The measurements of organs and body proportions studied included: (i) lineal biometric characters: body length (*BL*), body width (*BW*), perimeter (*P*), cone body length (*CL*), cone body width (*CW*), distance between the anterior end of the body and the acetabulum (*A-VS*), distance between the posterior end of the body and the acetabulum (*E-VS*), pharynx vertical diameter (*Ph ver. ø*), pharynx horizontal diameter (*Ph hor. ø*), and testes length (*T*); (ii) surfaces: body surface (*BS*), oral sucker surface (*OSS*), and ventral sucker surface (*VSS*); and (iii) ratios: body length/width (*BL/BW*), proportion between sucker surfaces (*OSS/VSS*), testes/body length (*T/BL*). The length of the tegumentary spines and length/width of the eggs were also studied. 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, USA).

Table 1. Comparative morphometric data of *Fasciola hepatica* populations from Bolivia and Spain.

Adults	Bolivia n = 112	Spain n = 53
BS (mm^2)	22.40–198.34 (79.73 ± 3.16)	59.40–197.24 (126.153 ± 3.30)
BL (mm)	9.39–26.13 (15.64 ± 0.30)	12.13–26.51 (19.68 ± 0.37)
BW (mm)	3.44–10.58 (7.07 ± 0.15)	6.87–10.82 (9.02 ± 0.11)
BL/BW	1.62–3.06 (2.25 ± 0.33)	1.13–2.95 (4.86 ± 0.56)
P (mm)	23.16–61.41 (37.49 ± 0.71)	31.24–61.32 (46.98 ± 0.76)
CL (mm)	1.37–3.00 (2.24 ± 0.03)	1.31–2.75 (1.94 ± 0.05)
CW (mm)	1.40–3.40 (2.30 ± 0.03)	2.29–3.71 (2.99 ± 0.05)
OSS (mm^2)	0.12–0.56 (0.33 ± 0.01)	0.21–1.03 (0.46 ± 0.02)
VSS (mm^2)	0.23–1.20 (0.70 ± 0.020)	0.66–1.89 (1.08 ± 0.04)
OSS/VSS	0.21–1.12 (0.50 ± 0.02)	0.04–0.87 (0.44 ± 0.01)
A–VS (mm)	1.30–3.09 (2.04 ± 0.03)	1.13–2.89 (2.02 ± 0.06)
E–VS (mm)	7.13–23.39 (12.64 ± 0.29)	8.23–22.35 (16.39 ± 0.35)
Ph ver. ø (mm)	0.51–0.97 (0.69 ± 0.04)	0.60–0.89 (0.73 ± 0.03)
Ph hor.ø (mm)	0.29–0.57 (0.37 ± 0.02)	0.34–0.49 (0.41 ± 0.02)
T (mm)	3.84–15.53 (8.80 ± 0.89)	6.15–15.84 (10.48 ± 1.27)
T/BL	0.33–0.55 (0.42 ± 0.02)	0.37–0.54 (0.47 ± 0.02)
Tegumentary spine size (μm)	14.20–59.40 (30.84 ± 3.04)	22.9–45.9 (30.57 ± 3.83)
Eggs	**n = 70**	**n = 90**
Length (μm)	126.30–149.30 (138.46 ± 0.54)	128.20–148.20 (137.11 ± 0.47)
Width (μm)	68.90–91.90 (76.62 ± 0.31)	68.40–99.70 (81.23 ± 0.39)

All values shown as range with mean ± SE, in parentheses. SE, standard error; n, sample size.

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Allometry

To study the relationship between two morphometric variables y_1 and y_2 in adult flukes, we employed: (i) the function proposed by Valero *et al*. (1996), obtained from a logistic model with respect to time:

$$(y_{2m} - y_2)/y_2 = c[(y_{1m} - y_1)/y_1]^b \quad (1)$$

where $y_1 = BS$ or BL, y_{1m} is the maximum value towards which y_1 tends, y_2 is one of the measurements analysed, y_{2m} is the maximum value towards which y_2 tends, and c and b are constants; (ii) the classical allometric expression (power model):

$$y = cx^b \quad (2)$$

where y = organ size, $x = BS$ or BL, b = allometric exponent and c is a constant.

BL and *BS* were selected as age measurements for the natural population, taking into account the general adult stage morphology of *F. hepatica*. For calculating y_m's 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: $tBS = ln[(BSmax - BS)/BS]$; $tBL = ln[(BLmax - BL)/BL]$; $tBW = ln[(BWmax - BW)/BW]$; $tP = ln[(Pmax - P)/P]$, and $tE\text{-}VS = ln[(E\text{-}VSmax - E\text{-}VS)/E\text{-}VS]$. Differences in allometric curves were sought by analysis of covariance (ANCOVA) (one-way analysis of variance design with one covariate) using initial *tBS* or *tBL* as a covariate. The growth rates of adults from the two populations were compared using the same y_m (the higher of the two populations). The effect-size measures are controlled by the eta-squared statistic (ETA), used to describe the proportion of the total variability 'explained' by the grouping or factor variable (Norusis, 1994).

Results

In the Bolivian material, characteristics useful for the identification of *Fasciola* spp. such as body shape and size, body length/width ratio, pharynx, tegumentary spine size, sucker ratio, ramification patterns in intestinal branches and testes, testes size, testes/body length ratio and egg size (table 1) are typically those of *F. hepatica* (Varma, 1953; Kendall, 1965; Kimura *et al*., 1984). Neither *F. gigantica* nor intermediate forms were identified.

Morphometric values of both Bolivian and Spanish material are shown in table 1. The allometric function obtained from a logistic model (table 2) with respect to time [1] (fig. 1A,B,C) fits better than the power model [2], as it can be deduced from: (i) each one of *BL, BW, P* and *E-VS* as a function of *BS*; and (ii) each one of *BW, P* and *E-VS* as a function of *BL*. Table 2 also provides the corresponding r^2. Significant differences (ANCOVA) ($P < 0.05$) in *tBW/tBS* and *tE-VS/tBS* between Bolivian and Spanish populations were not detected. Function allometry shape was significantly different between Bolivian and Spanish populations in *tBL/tBS* (ETA = 1%), *tP/tBS* (ETA = 0.5%), *tBW/tBL* (ETA = 11%), *tP/tBL* (ETA = 13%), and *tE-VS/tBL* (ETA = 21%).

Table 2. Allometric function[a] obtained from a logistic model with respect to time in adult worms of *Fasciola hepatica* from Bolivian and Spanish sheep.

y_1	y_2	y_{1m}	y_{2m}	b ± SE	c ± SE	r^2	sse allometric model	sse power model
Bolivian population								
BS	BL	200	27	0.636 ± 0.026	0.538 ± 0.011	0.88	129.531	(137.647)
BS	BW	200	10.6	1.008 ± 0.048	0.288 ± 0.012	0.85	40.814	(45.987)
BS	P	200	63	0.680 ± 0.018	0.490 ± 0.007	0.94	323.368	(335.867)
BS	E-VS	200	24.5	0.632 ± 0.028	0.706 ± 0.016	0.86	134.058	(147.567)
BL	BW	27	10.6	1.178 ± 0.118	0.694 ± 0.033	0.56	124.219	(128.018)
BL	P	27	63	1.004 ± 0.022	0.933 ± 0.009	0.96	207.515	(208.601)
BL	E-VS	27	24.5	0.986 ± 0.012	1.307 ± 0.008	0.98	11.277	(11.708)
Spanish population								
BS	BL	201	27.5	0.833 ± 0.065	0.608 ± 0.022	0.82	64.579	(64.843)
BS	BW	201	10.9	0.759 ± 0.103	0.300 ± 0.017	0.56	14.617	(14.640
BS	P	201	64	0.795 ± 0.042	0.543 ± 0.013	0.91	137.664	(138.426)
BS	E-VS	201	22.9	0.986 ± 0.077	0.650 ± 0.027	0.83	55.546	(55.960)
BL	BW	27.5	10.9	0.313 ± 0.041	0.449 ± 0.136	0.31	26.414	(26.415)
BL	P	27.5	64	0.873 ± 0.029	0.814 ± 0.021	0.96	57.133	(58.921)
BL	E-VS	27.5	22.9	1.163 ± 0.031	1.148 ± 0.031	0.97	7.618	(8.854)

sse comparison between the allometric[a] and the power[b] models (only pairs of variables showing significant fitness to both models are included) (b, c = constants; SE = standard error; r^2 = adjusted; y_m = maximum value of biometric characters in the allometric model; *sse* = least squares residual in both models).

[a] $(y_{2m} - y_2)/y_2 = c\,[(y_{1m} - y_1)/y_1]^b$

[b] $y = cx^b$

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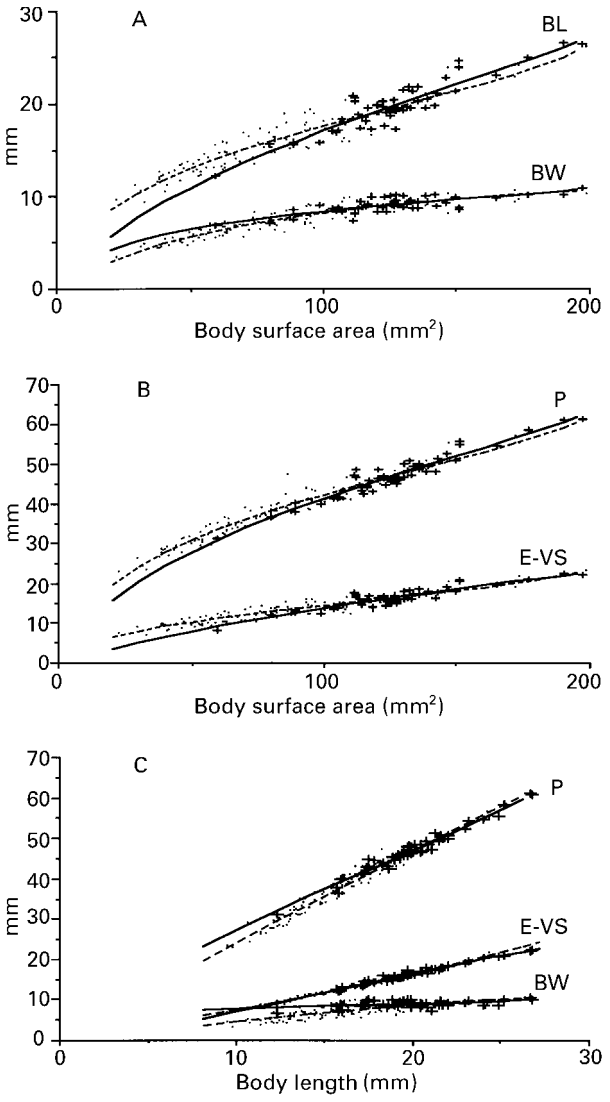

Fig. 1. Allometric model $(y_{2m}-y_2)/y_2 = c\,[(y_{1m}-y_1)/y_1]^b$ obtained in adult worms of *Fasciola hepatica* from naturally infected sheep in Bolivia (·) and Spain (+); each point represents an individual adult. A: changes in body length (*BL*) as a function of the body surface (*BS*), and body width (*BW*) as a function of the body surface (*BS*); B: perimeter (*P*) as a function of the body surface (*BS*), and distance between the posterior end of the body and the acetabulum (*E-VS*) as a function of the body surface (*BS*); C: body width (*BW*) as a function of body length (*BL*), perimeter (*P*) as a function of body length (*BL*), and distance between the posterior end of the body and the acetabulum (*E-VS*) as a function of body length (*BL*).

Discussion

Despite the geographic distance and the marked environmental differences between the Bolivian Altiplano and the Spanish regions concerned, the results do not show any differences in egg size, and in adults only a smaller size in the majority of the parameters in the Bolivian material is found. As size differences may be related to age (Dawes, 1962a,b; Valero *et al*., 1996, 1998), the discussion must refer only to results in the shape of the growth trajectory. All biometric characteristics analysed (fig. 1A,B,C) confirm that the two populations belong to the same species *F. hepatica*, though the Bolivian population shows a smaller morphometric development than the Spanish one. In any case, the differences in the shape of the allometries between both populations studied are only minor and are likely to be due to geographical variability related to altitude effects or genetic isolation. The existence of only slight significant morphometric differences is surprising when taking into account the great geographic distance existing between both liver fluke populations and the markedly different conditions imposed by the very high altitude. A comparison of geographically closer populations such as those of Spain and the island of Corsica showed pronouncedly larger differences (Marcos, 1993). This can be interpreted as the consequence of the Bolivian population being a recent isolation from Iberian populations. Molecular studies are presently underway to determine whether DNA information supports this phenotype assumption.

Our results show that the allometric model proposed by Valero *et al*. (1996) may be successfully applied to comparisons of digenean populations which follow a logistic model versus time, even in cases of parasites which present morphoanatomic characteristics that are difficult to analyse, as in the case of species of *Fasciola* (Kimura *et al*., 1984).

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