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Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS)

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Abstract The quantification of the different sizes and shapes of *Fasciola hepatica* and *Fasciola gigantica* from bovines has been achieved for the first time in natural allopatric populations. Linear measurements, areas and ratios of gravid adults and eggs of *F. hepatica* (from France and Spain) and *F. gigantica* (from Burkina Faso) were analysed using a computer image analysis system and an allometric model: $(y_{2m}-y_2)/y_2 = c\left[(y_{1m}-y_1)/y_1\right]^b$, where y_1=body area 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. All the measurements overlap in the two fasciolids, apart from the distance between the ventral sucker and the posterior end of the body, body roundness and body length/body width ratio. The results obtained may be useful in *Fasciola* species identification in countries where both species coexist.

Fasciolosis is a disease caused by two trematode species that belong to *Fasciola*: *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855. This disease has traditionally been thought of as a veterinary problem causing important economic losses due to its effect on farm animals, especially sheep and cattle (Boray 1982) and of only secondary impact on humans (Chen and Mott 1990). However, fasciolosis has been shown to be an important public health problem (Mas-Coma et al. 1999a,b), with human cases reported in countries on five continents (Esteban et al. 1998) and human endemic areas ranging from hypo- to hyperendemic (Mas-Coma et al. 1999b). In recent surveys, the estimated rate of worldwide human infection has oscillated between 2.4 (Rim et al. 1994) and 17 million (Hopkins 1992), while the population at risk is estimated to be 180 million (World Health Organization 1995). Due to the adaptation and colonization capacities of its causal agents and vector species, fasciolosis is a disease that has a very high potential for expansion. It is emerging or reemerging in many countries and its prevalences, intensities and geographical distribution are increasing (Mas-Coma 2004a,b). Today, fasciolosis is the vector-borne parasitic disease presenting the widest latitudinal, longitudinal and altitudinal distribution known (Mas-Coma et al. 2003).

Morphology has been the most frequently used criterion for systematic studies on *Fasciola* flukes. The different species and/or subspecies originally described in the literature were differentiated through the analysis of adult and egg samples from domestic animal hosts; most of these were later invalidated giving rise to the present situation in which only two species are accepted (Kendall 1965). The differences most frequently highlighted between the species are the greater length of *F. gigantica* and the general appearance of the body (Kendall 1965). Other authors have differentiated the species on the basis of the ramification patterns of the reproductive organs and intestines (Jackson 1921; Watanabe 1962; Bergeon and Laurent 1970), but the natural branching shape of these structures make this characteristic inconvenient. Although many morphometric studies have dealt with the study of *F. hepatica*, very few have focused on *F. gigantica* (Srimuzipo et al. 2000), and even fewer studies have focused on the comparison of both species.

However, today, it is known that fasciolid species differentiation in areas where both species overlap becomes a very complex task. First, literature did not follow measurement standardization, and therefore, results cannot be compared. Second, allometric growth was not taken into account in those studies. Third, recent exhaustive morphometric comparisons of adults and eggs of natural liver fluke populations from different host species and adults and eggs experimentally obtained in Wistar rats infected with isolates from different natural hosts revealed that the

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definitive host species decisively influences the size of adults and eggs and that, this influence does not persist in a heterologous host (Valero et al. 2001b). Although classical morphometrical measurements of adult specimens and eggs of both species in the different revisions conducted up to date clearly demonstrate an overlap, conclusions cannot be obtained because parasite materials used are mixtures from different host species and measurements are not separated accordingly.

Fourth, in overlapping areas, intermediate forms usually appear which finally remain classified as *Fasciola* sp. because of the impossibility to ascribe them to one species or the other. This overlapping distribution of both species has become the basis of a long ranging controversy on the taxonomic identity of the *Fasciola* species found in Asian countries, especially Japan, Taiwan, the Philippines and Korea, in which a wide range of morphological types has been detected, including hybrids (Terasaki et al. 1982; Adlard et al. 1993; Agatsuma et al. 1994; Hashimoto et al. 1997; Itagaki and Tsutsumi 1998; Itagaki et al. 1998).

For a more precise morphometric study of both species, only adult flukes found in naturally infected bovines were used. Materials from continental (Spain) and insular (Corsica) origins were used for *F. hepatica*, considering Europe to be the origin of this fasciolid (Mas-Coma et al. 2003). Specimens from Burkina Faso were used as representatives of *F. gigantica*, as *Radix natalensis* is the only lymnaeid species (no *Galba/Fossaria*) in that country (Bargues et al. 2005; Bargues and Mas-Coma 2005) and as *F. gigantica* was originally described from a *Giraffa camelopardalis* from Sub-Saharan Africa found in a travelling menagerie in England (Cobbold 1855) and the same fasciolid species was somewhat later re-described originating from cattle in Senegal (Railliet 1895). The aims

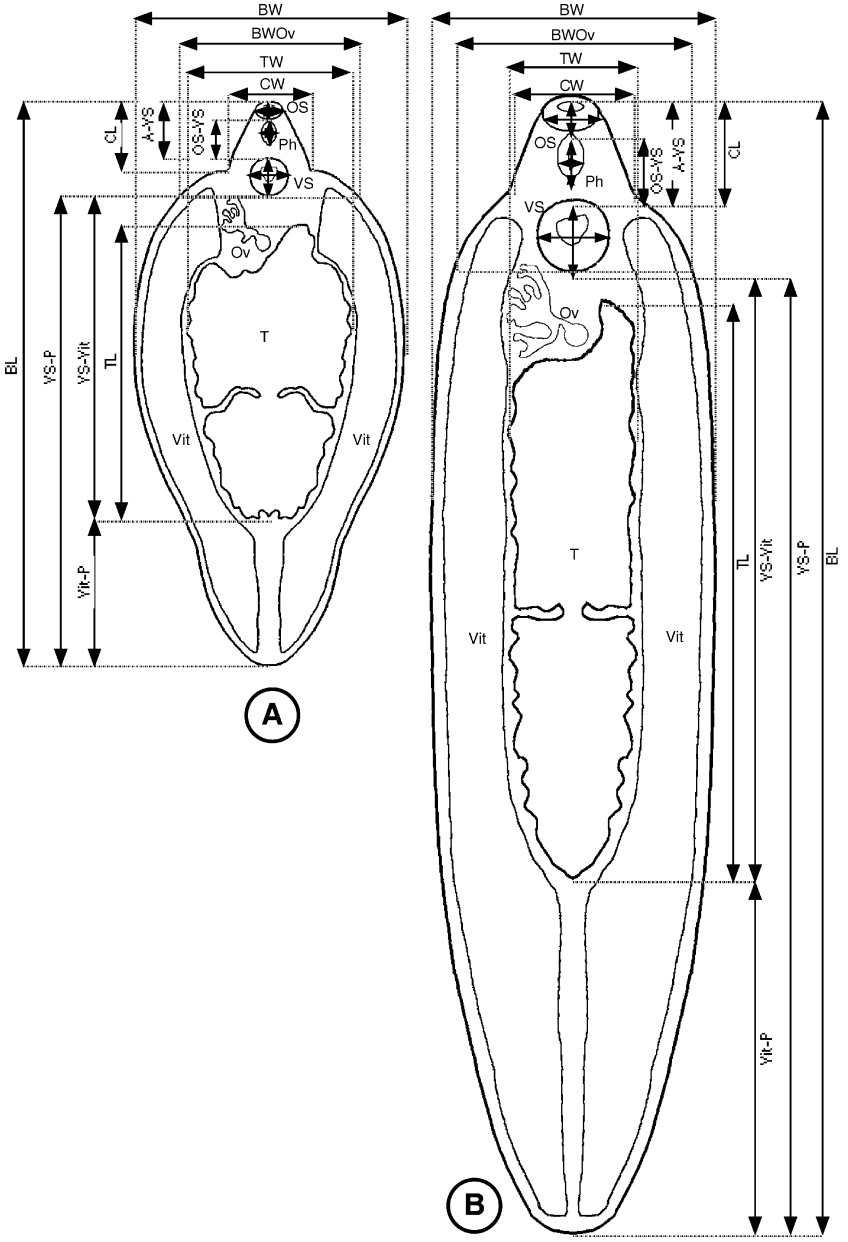

Fig. 1 Standardized measurements in gravid adults:
a *Fasciola hepatica*;
b *Fasciola gigantica*

of the present study are 1) to characterize the morphometry of the adult stage of *F. gigantica* from bovines in natural liver fluke populations from Burkina Faso by means of computer image analysis and the use of an allometric model; 2) to morphometrically compare adults of the two species of *Fasciola* from the above-mentioned allopatric populations to prove they can be differentiated.

Materials and methods

Parasites

All liver fluke materials were obtained from the bile ducts of naturally infected bovines: 84 specimens from the Mercavalencia slaughterhouse (Valencia, Spain); 86 specimens from the Portovechio slaughterhouse (Corsica, France); 81 specimens from the Bobo-Dioulasso slaughterhouse (Burkina Faso). All fasciolid specimens included in the study were gravid adult flukes, ranging from slightly gravid to completely gravid. Adult worms were fixed in Bouin's solution between a slide and coverglass but without coverglass pressure to avoid distortion. Then, the specimens were stained with Grenacher's borax, differentiated, dehydrated and mounted in Canada balsam.

Eggs were measured directly from the final part of the uterus of the adult specimens that were included in the morphometric study. Eggs were selected based on the level of development (only fully mature, dark brownish eggs were included), the intactness of the shell (shell wall well-formed), and the appropriate position for image capture (to assure that egg length was correctly measured). A total of 356 eggs was studied, 113 from Valencia, 101 from Corsica and 142 from Bobo-Dioulasso.

Measurement techniques

All standardized measurements of adults were made according to methods proposed by Valero et al. (1996) for Fasciolidae. The measurements of organs and body proportions studied included (Fig. 1):

(1) lineal biometric characters: body length (BL), maximum body width (BW), body width at ovary level (BWOv), body perimeter (BP), body roundness (BR), cone length (CL), cone width (CW), maximum diameter of the oral sucker (OS max), minimum diameter of the oral sucker (OS min), maximum diameter of the ventral sucker (VS max), minimum diameter of the ventral sucker (VS min), distance between the anterior end of the body and the ventral sucker (A-VS), distance between the oral sucker and the ventral sucker (OS-VS), distance between the ventral sucker and the union of the vitelline glands (VS-Vit), distance between the union of the vitelline glands and the posterior end of the body (Vit-P), distance between the ventral sucker and the posterior end of the body (VS-P), pharynx length (PhL), pharynx width (PhW), testicular space (taking both testes together—see Fig. 1) length (TL), testicular space width (TW) and testicular space perimeter (TP);
(2) areas: body area (BA), oral sucker area (OSA), ventral sucker area (VSA), pharynx area (PhA) and testicular space area (TA);
(3) ratios: body length over body width (BL/BW), body width at ovary level over cone width (BWOv/CW), oral sucker area over ventral sucker area (OSA/VSA) and body length over the distance between the ventral sucker and the posterior end of the body (BL/VS-P).

Egg characteristics studied were the following:

(1) lineal biometric characters: egg length (EL), egg width (EW), egg perimeter (EP) and egg roundness (ER);
(2) areas: egg area (EA);
(3) ratios: egg length over egg width (EL/EW).

The body roundness ($BR=BP^2/4\pi BA$) and egg roundness ($ER=EP^2/4\pi EA$) measurements were used to quantify the body shape and egg shape, respectively. It is a measurement of how circular an object is (the expected perimeter of a circular object divided by the actual perimeter). A circular object will have a roundness of 1.0, while more irregular objects will have larger values (Anonymous 2001). The measurements were made with a microscope (Leitz Dialux 20 EB) and using image analysis software (ImagePro Plus, version 4.5 for Windows, Media Cybernetics, Silver Spring, USA) for images captured by a digital camera (Nikon Coolpix 5400).

Allometry

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 and Hughes 1964; Valero et al. 1996, 1998, 2002) 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 can be calculated (Valero et al. 1991; Valero et al. 1999, 2001a,b).

To study the relationship between two morphometric variables, y_1 and y_2, in adult flukes, the function proposed by Valero et al. (1996, 1999) was employed. It is an alternative allometric function for *F. hepatica* adults based on logistic growth laws (variable differential growth rates) vs time:

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

where: y_1=BL or BA; y_{1m} is the maximum value towards which y_1 tends; y_2=the 30 measurements listed in Table 1; y_{2m} is the maximum value towards which y_2 tends; and b

and c are constants. BL and BA were selected as age measurements for the natural population, taking into account the general adult stage morphology of *F. hepatica* and *F. gigantica*. The function was adjusted to these 55 pairs of variables, of which 23 showed a significant adjustment in at least two of the three populations studied using MacCurveFit version 1.0.1:

(1) BW vs BL, BWOv vs BL, BP vs BL, A-VS vs BL, VS-Vit vs BL, Vit-P vs BL, VS-P vs BL, TA vs BL, TL vs BL, TW vs BL, and TP vs BL;
(2) BL vs BA, BW vs BA, BWOv vs BA, BP vs BA, A-VS vs BA, VS-Vit vs BA, Vit-P vs BA, VS-P vs BA, TA vs BA, TL vs BA, TW vs BA, and TP vs BA.

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

Statistical data and analyses

Data processing was carried out with SPSS software version 12.0 for Windows and based on the methodology by Valero et al. (1999). Adjusted nonlineal curves were tested using R^2 and sse.

For the comparison of allometric curves, log e transformations were necessary:

(1) lineal biometric characters: $tBL=\ln[(BL_{max}-BL)/BL]$; $tBW=\ln[(BW_{max}-BW)/BW]$; $tBWOv=\ln[(BWOv_{max}-BWOv)/BWOv]$; $tBP=\ln[(BP_{max}-BP)/BP]$; tBR=ln

Table 1 Comparative morphometric data of liver flukes from bovines: *Fasciola hepatica* populations from Spain and Corsica and *Fasciola gigantica* from Burkina Faso

Adult measurements (mm)	Spain, n=84	Corsica, n=86	Burkina Faso, n=81
Body length, BL	11.64–22.93 17.41±0.23	12.22–29.00 20.45±0.37	28.82–52.30 39.72±0.58
Body width, BW	6.41–13.88 10.02±0.17	4.88–14.07 10.71±0.18	6.03–11.84 8.45±0.14
BW at ovary level, BWOv	4.53–11.67 7.90±0.14	4.46–11.46 8.06±0.15	5.33–11.55 7.59±0.13
Body perimeter, BP	28.58–54.40 43.05±0.55	30.21–66.43 49.21±0.81	63.19–113.71 85.25±1.21
Body roundness, BR	1.06–1.58 1.23±0.01	1.10–1.55 1.25±0.01	1.71–3.65 2.47±0.05
Cone length, CL	1.09–2.92 2.02±0.04	1.58–3.04 2.21±0.03	2.10–3.36 2.67±0.04
Cone width, CW	2.35–4.21 3.20±0.04	2.30–4.01 3.08±0.04	2.23–5.08 3.74±0.06
OS maximum diameter, OS_{max}	0.57–1.03 0.85±0.01	0.60–0.99 0.84±0.01	0.52–1.17 0.83±0.02
OS minimum diameter, OSmin	0.44–0.77 0.61±0.01	0.40–0.83 0.65±0.01	0.21–0.95 0.62±0.02
VS maximum diameter, VS_{max}	0.92–1.49 1.13±0.01	0.69–1.38 1.10±0.01	0.87–1.92 1.50±0.02
VS minimum diameter, VSmin	0.86–1.35 1.12±0.01	0.83–1.26 1.08±0.01	0.80–1.83 1.38±0.02
Distance between anterior end of body and VS, A-VS	1.12–2.92 2.05±0.04	1.35–3.04 2.48±0.04	1.46–3.01 2.36±0.03
Distance between suckers, OS-VS	0.57–2.41 1.42±0.04	0.89–2.32 1.82±0.03	1.15–2.22 1.71±0.03
Distance between VS and union of vitelline glands, VS-Vit	6.57–14.31 9.60±0.17	5.97–18.22 11.64±0.26	12.26–34.11 22.68±0.45
Distance between Vit and posterior end of body, Vit-P	2.63–7.57 4.73±0.10	2.90–8.99 5.17±0.15	8.97–21.43 13.45±0.32
Distance between VS and P, VS-P	9.51–19.94 14.40±0.22	8.86–25.08 16.90±0.36	26.28–50.09 36.39±0.59
Pharynx length, PhL	0.52–1.00 0.70±0.01	0.10–0.97 0.76±0.01	0.46–1.06 0.78±0.02
Pharynx width, PhW	0.26–0.83 0.44±0.01	0.29–0.63 0.41±0.01	0.23–0.68 0.42±0.01
Testicular space length, TL	5.45–11.68 7.91±0.15	5.38–15.99 9.85±0.23	12.76–29.38 18.76±0.38
Testicular space width, TW	4.18–8.93 6.61±0.12	3.17–10.11 7.39±0.14	3.24–8.66 5.51±0.12
Testicular space perimeter, TP	17.10–34.34 25.32±0.42	15.75–40.29 29.85±0.56	25.97–68.06 44.62±0.87
Body area, BA	54.90–197.40 123.83±3.28	46.80–261.71 153.61±4.70	162.58–482.91 249.38±7.48
Oral sucker area, OSA	0.25–0.56 0.41±0.01	0.22–0.59 0.43±0.01	0.10–1.11 0.46±0.03
Ventral sucker area, VSA	0.67–1.57 0.99±0.02	0.45–1.30 0.93±0.02	0.56–3.52 1.86±0.08
Pharynx area, PhA	0.16–0.57 0.31±0.01	0.04–0.54 0.31±0.01	0.12–0.64 0.33±0.02
Testicular space area, TA	17.85–70.62 40.45±1.24	13.77–100.47 56.30±1.96	41.05–187.50 80.75±3.44
BL/BW ratio	1.29–2.77 1.74±0.03	1.33–2.80 1.91±0.03	3.40–6.77 4.70±0.08
BWOv/CW ratio	1.52–3.46 2.47±0.04	1.47–3.86 2.62±0.05	1.34–3.63 2.03±0.05
Sucker ratio, OSA/VSA	0.24–0.60 0.41±0.01	0.21–0.99 0.46±0.01	0.08–0.40 0.25±0.01
BL/VS-P ratio	1.14–1.31 1.21±0.004	0.95–1.38 1.21±0.01	0.95–1.20 1.09±0.004

All values are shown as range with mean±SE
SE Standard error, n sample size

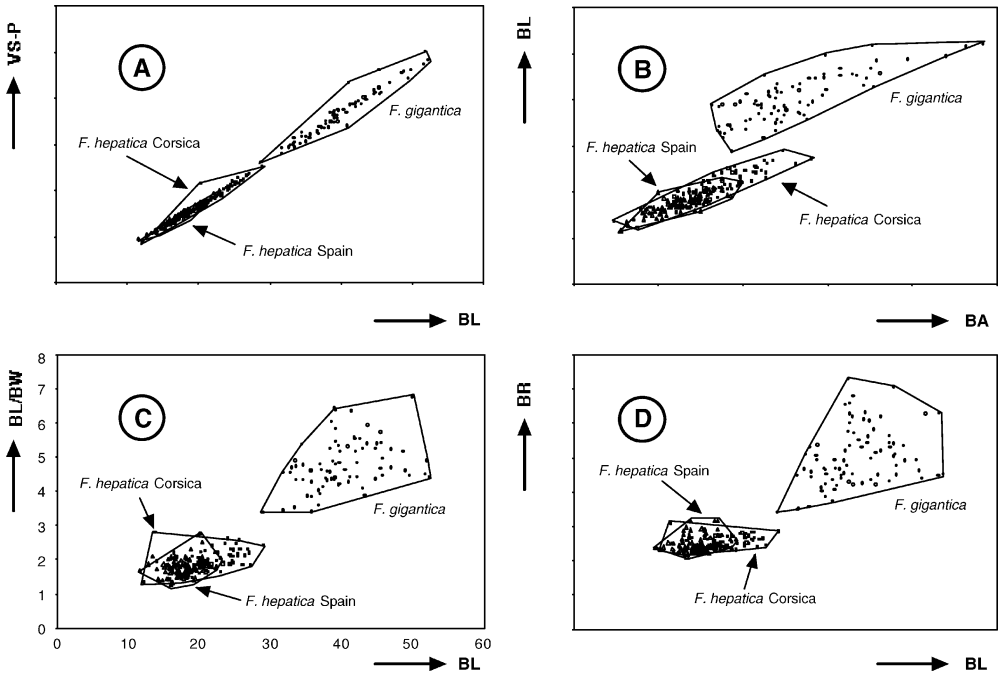

Fig. 2 Changes in different parameters measured in adult worms of *Fasciola* from naturally infected bovines from Spain, Corsica and Burkina Faso, as a function of either body length (*BL*) or body area (*BA*). Each point represents an adult individual (Δ=*F. hepatica* from Spain; □=*F. hepatica* from Corsica; O=*F. gigantica* from Burkina Faso). **a** body length/body width ratio (*BL*/*BW*) in function of BL; **b** body roundness (*BR*) in function of BL; **c** distance between the ventral sucker and the posterior end of the body (*VS-P*) in function of *BL*; **d** *BL* in function of *BA*

$[(BR_{max}-BR)/BR]$; $tCL=\ln[(CL_{max}-CL)/CL]$; $tCW=\ln[(CW_{max}-CW)/CW]$; $tOS_{max}=\ln[((OS_{max})_{max}-OS_{max})/OS_{max}]$; $tOS_{min}=\ln[((OS_{min})_{max}-OS_{min})/OS_{min}]$; $tVS_{max}=\ln[((VS_{max})_{max}-VS_{max})/VS_{max}]$; $tVS_{min}=\ln[((VS_{min})_{max}-VS_{min})/VS_{min}]$; $tA\text{-}VS=\ln[(A\text{-}VS_{max}-A\text{-}VS)/A\text{-}VS]$; $tOS\text{-}VS=\ln[(OS\text{-}VS_{max}-OS\text{-}VS)/OS\text{-}VS]$;

Table 2 Allometric function[a] obtained from a logistic model with respect to time in *Fasciola hepatica* adults obtained from bovines from Spain

y_1	y_2	y_{1m}	y_{2m}	c±SE	b±SE	R^2	sse
BL	BW	23.21	13.88	NS	NS	0.20	153.42
BL	BWOv	23.21	11.67	NS	NS	0.10	121.23
BL	BP	23.21	54.40	0.871±0.044	1.102±0.051	0.89	221.17
BL	A-VS	23.21	2.92	NS	NS	0.08	9.45
BL	VS-Vit	23.21	14.31	1.377±0.091	0.937±0.060	0.81	39.22
BL	Vit-P	23.21	7.57	1.018±0.122	0.463±0.098	0.26	51.77
BL	VS-P	23.21	20.00	1.151±0.032	0.979±0.026	0.96	12.27
BL	TA	23.21	70.62	2.539±0.306	1.142±0.106	0.68	3,205.79
BL	TL	23.21	12.31	1.411±0.100	0.837±0.062	0.77	36.75
BL	TW	23.21	9.93	NS	NS	0.18	76.89
BL	TP	23.21	34.34	1.041±0.094	0.987±0.085	0.70	344.23
BA	BL	197.42	23.21	0.454±0.017	0.578±0.051	0.66	121.05
BA	BW	197.42	13.88	0.574±0.020	0.755±0.050	0.78	41.87
BA	BWOv	197.42	11.67	0.655±0.300	0.621±0.061	0.58	56.77
BA	BP	197.42	54.40	0.395±0.007	0.789±0.028	0.93	146.25
BA	A-VS	197.42	2.92	NS	NS	0.06	9.63
BA	VS-Vit	197.42	14.31	0.690±0.005	−0.018±0.002	0.28	152.11
BA	Vit-P	197.42	7.57	NS	NS	0.13	60.60
BA	VS-P	197.42	20.00	0.531±0.021	0.575±0.054	0.63	115.82
BA	TA	197.42	70.62	1.188±0.051	0.899±0.063	0.82	1,776.13
BA	TL	197.42	12.31	0.713±0.036	0.465±0.063	0.47	82.40
BA	TW	197.42	9.93	0.532±0.026	0.834±0.073	0.65	32.78
BA	TP	197.42	34.34	0.522±0.019	0.753±0.054	0.76	284.60

c, *b* Constants, *SE* standard error, R^2 adjusted, y_m maximum value of biometric characters in the allometric model, *sse* least square residuals, *NS* not significant

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

Table 3 Allometric function[a] obtained from a logistic model with respect to time in *Fasciola hepatica* adults obtained from bovines from Corsica

y_1	y_2	y_{1m}	y_{2m}	c±SE	b±SE	R^2	sse
BL	BW	29.00	14.66	0.570±0.056	0.532±0.097	0.26	161.01
BL	BWOv	29.00	11.46	NS	NS	0.10	133.55
BL	BP	29.00	66.49	0.835±0.022	1.100±0.031	0.95	228.69
BL	A-VS	29.00	3.04	0.426±0.044	0.716±0.117	0.33	5.38
BL	VS-Vit	29.00	18.22	1.258±0.064	0.920±0.052	0.84	78.95
BL	Vit-P	29.00	9.36	1.635±0.131	0.782±0.074	0.66	58.03
BL	VS-P	29.00	25.51	1.242±0.030	1.018±0.026	0.97	33.12
BL	TA	29.00	107.33	2.179±0.179	1.048±0.079	0.76	7,050.03
BL	TL	29.00	15.99	1.367±0.073	0.903±0.054	0.82	70.41
BL	TW	29.00	10.35	0.620±0.067	0.542±0.107	0.24	104.69
BL	TP	29.00	40.66	0.868±0.056	1.534±0.077	0.77	521.14
BA	BL	280.50	29.00	0.484±0.014	0.779±0.048	0.81	191.56
BA	BW	280.50	14.66	0.416±0.014	0.715±0.054	0.73	59.99
BA	BWOv	280.50	11.46	0.469±0.023	0.605±0.076	0.42	86.07
BA	BP	280.50	66.49	0.409±0.007	0.853±0.027	0.94	278.40
BA	A-VS	280.50	3.04	0.256±0.015	0.639±0.092	0.35	5.19
BA	VS-Vit	280.50	18.22	0.641±0.026	0.721±0.065	0.64	172.34
BA	Vit-P	280.50	9.36	0.930±0.048	0.659±0.077	0.54	77.04
BA	VS-P	280.50	25.51	0.592±0.020	0.796±0.057	0.76	229.56
BA	TA	280.50	107.33	1.093±0.028	1.057±0.043	0.92	2,204.55
BA	TL	280.50	15.99	0.710±0.028	0.731±0.062	0.68	123.66
BA	TW	280.50	10.35	0.451±0.019	0.738±0.066	0.66	46.68
BA	TP	280.50	40.66	0.418±0.012	0.950±0.048	0.87	296.38

c, b Constants, *SE* standard error, R^2 adjusted, y_m maximum value of biometric characters in the allometric model, *sse* least square residuals, *NS* not significant

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

tVS-Vit=ln[(VS-Vit$_{max}$−VS-Vit)/VS-Vit]; tVit-P=ln[(Vit-P$_{max}$−Vit-P)/Vit-P]; tVS-P=ln[(VS-P$_{max}$−VS-P)/VS-P]; tPhL=ln[(PhL$_{max}$−PhL)/PhL]; tPhW=[(PhW$_{max}$−PhW)/PhW]; tTL=ln[(TL$_{max}$−TL)/TL]; tTW=ln[(TW$_{max}$−TW)/TW]; and tTP=ln[(TP$_{max}$−TP)/TP] (Valero et al. 1999, 2001b).

(2) areas: tBA=ln[(BA$_{max}$−BA)/BA]; tOSA=ln[(OSA$_{max}$−OSA)/OSA]; tVSA=ln[(VSA$_{max}$−VSA)/VSA]; tPhA=ln[(PhA$_{max}$−PhA)/PhA]; and tTA=ln[(TA$_{max}$−TA)/TA];

(3) ratios: t(BL/BW)=ln[(((BL/BW)$_{max}$)−(BL/BW))/(BL/BW)]; t(BWOv/CW)=ln[(((BWOv/CW)$_{max}$)−(BWOv/CW))/(BWOv/CW)]; t(OSA/VSA)=ln[(((OSA/VSA)$_{max}$)−(OSA/VSA))/(OSA/VSA)]; and t(BL/VS-P)=ln[(((BL/VS-P)$_{max}$)−(BL/VS-P))/(BL/VS-P)].

Differences in allometric curves were sought by analysis of covariance (ANCOVA) (one-way analysis of variance design with one covariable) using initial tBL and tBA as a covariable. The growth rates of adults from the three populations were compared using the same y_m (the highest of the three populations). The effect–size measures are controlled by the eta-squared statistic (ETA) and power (Norusis 1994). The usefulness of the measurements obtained for the specific differentiation of each species was also studied using discriminant analysis by using the geographic area of origin as the grouping variable. The stepwise regression procedure was applied using Wilks' lambda. Comparison of the average body shape measurements (BL/BW and BR) and the average egg measurements (EL, EW, EP, EA, EL/EW) from the different populations was carried out using the one-way ANOVA and three post hoc tests with different conservative approaches (Tukey's HSD, Scheffé and Bonferroni tests) (Sokal and Rohlf 1981). Values were considered statistically significant when $P<0.05$.

Results

Fluke size

The morphometric values of *F. hepatica* from Spain and Corsica and *F. gigantica* from Burkina Faso are shown in Table 1. The comparison of the morphometric results obtained shows that all the specimens from Burkina Faso are typical *F. gigantica* forms; neither *F. hepatica* nor intermediate forms were found in this African country. The comparison of the measurements in both species shows that VS-P (Fig. 2a) does not overlap in the materials studied (8.86–25.08 mm in *F. hepatica*; 26.28–50.09 mm in *F. gigantica*). Nevertheless, small *F. gigantica* adults with only very few eggs in the uterus (unfortunately not available in this study) may slightly overlap with the

Table 4 Allometric function[a] obtained from a logistic model with respect to time in *Fasciola gigantica* adults obtained from bovines from Burkina Faso

y_1	y_2	y_{1m}	y_{2m}	c±SE	b±SE	R^2	sse
BL	BW	52.63	11.84	0.618±0.070	0.374±0.090	0.26	97.67
BL	BWOv	52.63	11.55	0.743±0.072	0.329±0.074	0.27	87.39
BL	BP	52.63	114.00	0.951±0.013	0.914±0.013	0.99	90.08
BL	A-VS	52.63	3.01	0.463±0.058	0.454±0.106	0.26	4.92
BL	VS-Vit	52.63	34.11	1.164±0.099	0.735±0.074	0.67	417.40
BL	Vit-P	52.63	21.43	1.192±0.145	0.622±0.101	0.38	401.71
BL	VS-P	52.63	50.09	1.121±0.039	0.968±0.033	0.95	111.10
BL	TA	52.63	187.50	3.037±0.372	0.732±0.091	0.56	33,717.19
BL	TL	52.63	29.38	1.249±0.104	0.691±0.071	0.63	330.60
BL	TW	52.63	8.66	0.895±0.099	0.394±0.086	0.28	65.06
BL	TP	52.63	68.05	1.069±0.981	0.618±0.077	0.55	2,166.87
BA	BL	483.06	52.63	0.337±0.013	0.803±0.091	0.63	791.71
BA	BW	483.06	11.84	0.415±0.014	0.895±0.081	0.72	36.49
BA	BWOv	483.06	11.55	0.530±0.016	0.694±0.068	0.70	36.08
BA	BP	483.06	114.00	0.351±0.011	0.790±0.078	0.70	2,830.09
BA	A-VS	483.06	3.01	0.278±0.014	0.818±0.124	0.43	3.78
BA	VS-Vit	483.06	34.11	0.515±0.025	0.488±0.101	0.34	831.26
BA	Vit-P	483.06	21.43	0.596±0.034	0.602±0.124	0.24	498.22
BA	VS-P	483.06	50.09	0.389±0.016	0.716±0.099	0.52	1,055.98
BA	TA	483.06	187.50	1.413±0.037	1.151±0.059	0.90	7,743.81
BA	TL	483.06	29.38	0.585±0.023	0.706±0.088	0.54	418.61
BA	TW	483.06	8.66	0.594±0.018	0.886±0.074	0.76	22.01
BA	TP	483.06	68.05	0.542±0.022	0.681±0.090	0.53	2,252.95

c, b Constants, *SE* standard error, R^2 adjusted, y_m maximum value of biometric characters in the allometric model, *sse* least square residuals

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

highest values attributable to very gravid and old *F. hepatica* adults. It is important to note that although BL seemed to overlap between the species, this was due to the presence of only one *F. gigantica* specimen from Burkina Faso that presented a strong contraction due to improper fixation. The range of BL in *F. gigantica* without this specimen was 31.73–52.29 mm, while it was 12.22–29.00 mm in *F. hepatica* (Fig. 2b). Therefore, BL may also be considered useful for the specific differentiation when working with specimens that have been properly fixed although the same above-mentioned consideration noted for VS-P should also be taken into account for BL.

The characteristic size of each adult species is reflected in the maximum values (y_m) detected in the allometric functions. Tables 2, 3 and 4 give the y_m of the allometric functions (Eq. 1) obtained for the 23 pairs of variables studied for each adult population and provide the corresponding nonlinear regressions (R^2) and sse. The maximum values detected for BW, BWOv, A-VS and TW were higher in *F. hepatica* adults, while the rest of the values for y_m were higher in *F. gigantica* adults.

The stepwise discriminant analysis run to examine which variables best separate the two species from the three geographical areas revealed a combination of 12 variables (BL/BW, VS_{max}, A-VS, BR, VS_{min}, Vit-P, CW, TW, OS_{max}, BWOv, BP and BA). Both canonical discriminant functions (CDF) had statistically significant (P>0.001) values of Wilks' lambda, 0.022 and 0.523, respectively. The discriminant linear functions (axes 1, y_1; and 2, y_2) clearly differentiate the species (Fig. 3). This analysis yielded a 100% correct identification of the species in all three populations.

Fluke shape

To assess the possible usefulness of the ratios (BL/BW, BWOv/CW, OSA/VSA, and BL/VS-P), their increase or decrease with age was analysed. The adjustments of these ratios vs BL and BA were performed (data not shown). Interestingly, none of the adjustments were significant as there is no variability with age. Out of all the ratios studied, BL/BW was the only one that did not overlap between the species (1.29–2.80 in *F. hepatica*; 3.40–6.78 in *F. gigantica*) (Fig. 2c).

BR did not show an adjustment neither with BL nor with BA, i.e. it did not increase when BL and BA increased. This measurement (BR) did not overlap between the two species (1.06–1.58 in *F. hepatica*; 1.71–3.65 in *F. gigantica*) (Fig. 2d). Significant differences (ANOVA) (P<0.05) were detected in BL/BW and BR between the three populations analysed. In the comparison of BL/BW and BR using the Tukey's HSD, Scheffé and Bonferroni tests, significant differences were obtained in all *F.*

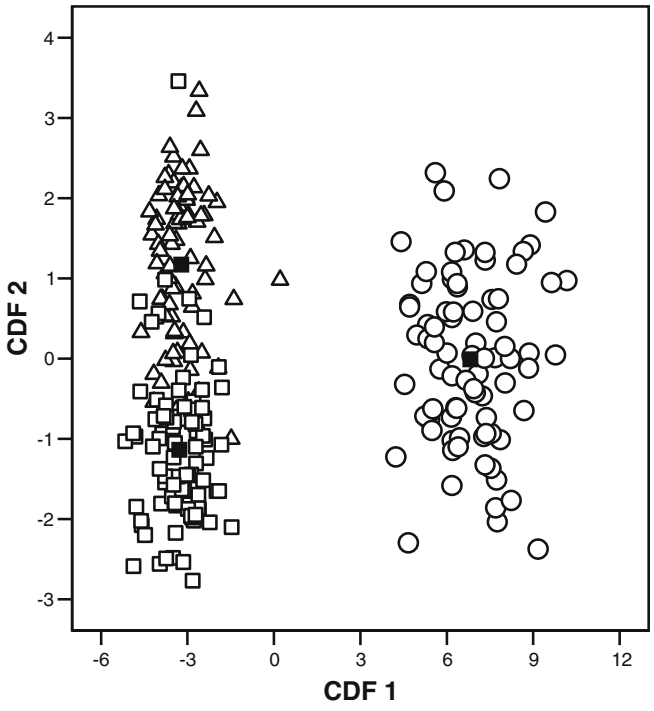

Fig. 3 Scatterplot of the discriminant scores of specimens belonging to the two species of *Fasciola*. *CDF1*: y_1=−1.335*BA*+1.738*BP*−0.181*A-VS*+0.237*Vit-P*−0.211OS_{max}+0.219VS_{min}+0.782VS_{max}+0.053*BL/BW*−0.391*TW*+0.054*BR*−0.153*CW*+0.012*BWOv* (Characters most strongly associated with this function: *BL/BW*, *VS-P*, *BL*, *BP*, *BR*, *Vit-P*, *VS-Vit*, *TL*, *OSA/VSA*, *BL/VS-P*, VS_{max}, VS_{min}, *VSA* and *CW*). *CDF2*: y_2=0.637*BA*−0.985*BP*−0.867*A-VS*+0.084*Vit-P*+0.378OS_{max}+0.679VS_{min}−0.294VS_{max}−0.489*BL/BW*−0.731*TW*+0.887*BR*+0.409*CW*+0.614*BWOv* (Characters most strongly associated with this function: *A-VS*, *OS-VS*, *TP*, *TA*, *TW*, *BA*, *CL*, *BW*, OS_{min}, *PhL*, *BWOv/CW*, *PhA*, *OSA*, *PhW*, *BWOv* and OS_{max}). Each point represents an adult individual (Δ=*F. hepatica* from Spain; □=*F. hepatica* from Corsica; O=*F. gigantica* from Burkina Faso). Canonical discriminant functions *CDF1* and *CDF2* derived using 251 specimens and 12 variables

gigantica–*F. hepatica* pairs, while no significant differences were detected in the *F. hepatica* pairs.

Tables 2, 3 and 4 give the parameters (*b*, *c*) of the allometric functions (Eq. 1) obtained for the 23 pairs of variables studied for each adult population. To establish the specific shape differences, the comparison between the material from *F. hepatica* (Spain and Corsica as one) and *F. gigantica* (Burkina Faso) was performed. No significant differences (ANCOVA) (P<0.05) in tBWOv vs tBL, tBL vs tBA, tBP vs tBA, and tOS_{max} vs tBA were detected (Table 5).

Moreover, significant interspecific differences between *F. hepatica* and *F. gigantica* populations were evident in 11 of them: tBW vs tBL, tA-VS vs tBL, tVS-Vit vs tBL, tVS-P vs tBL, tTA vs tBL, tTL vs tBL, tTW vs tBL, tTP vs tBL, tBW vs tBA, tTA vs tBA, and tTP vs tBA.

The function allometries that were significantly different are listed in Table 5 along with their corresponding ETA and power values (po). In view of these results, comparisons between each population of *F. hepatica* and that of *F. gigantica* were performed. To establish the influence of the geographical location on the intraspecific variability of *F. hepatica*, the material from Spain and Corsica was also compared. Out of the 23 pairs of variables studied (Table 5), significant intraspecific differences between the two *F. hepatica* populations were evident in five of them (tBL vs tBA, tBWOv vs tBA, tBP vs tBA, tVS-P vs tBA, and tTW vs tBA). These intraspecific differences were all related to the body area (BA) and were possibly due to the older age of the Corsican samples as the host animals from Corsica were older than the ones from Valencia.

Egg size and shape

The morphometric values of *F. hepatica* and *F. gigantica* eggs from the three populations studied are shown in Table 6. No significant differences (ANOVA) (P<0.05) were detected in EL, EP and EA when comparing *F. hepatica* pairs, while significant differences were found when comparing *F. hepatica* (Spain)–*F. gigantica* and *F. hepatica* (Corsica)–*F. gigantica* pairs. In EW, significant differences were observed when comparing *F. hepatica* pairs and the two above-mentioned *F. hepatica*–*F. gigantica* pairs. In EL/EW, significant differences were observed when comparing *F. hepatica* pairs and the eggs of the specimens from Spain and Burkina Faso, but no significant differences were observed when comparing the eggs of the specimens from Corsica and Burkina Faso.

Discussion

Adult and egg morphology

F. gigantica was originally differentiated from *F. hepatica* by its larger size (1.5–3.0 in.=34.5–69.0 mm), elongated body, the posterior end only beginning to narrow at a very short distance from the tail end (whereas the posterior end of *F. hepatica* presents a more V-like shape) and by the presence of numerous secondary intestinal ramifications (Cobbold 1855; see also Varma 1953.) Later, more samples of *F. gigantica* were described in other countries such as Senegal (Railliet 1895) and Egypt (Looss 1896). Much emphasis was placed on the external morphology, especially on the size and shape of the body. However, many authors agree that it is very difficult to achieve a precise classification as there are many variations in their morphological characteristics (Kimura et al. 1984).

In the present study, the quantification of the different sizes and shapes of *F. hepatica* and *F. gigantica* gravid adults and eggs in the same host (bovines) has been achieved for the first time in allopatric populations. The differences in shapes detected between both species follow the classical criterion described by Jackson (1921). Results here obtained are expected to furnish the basis on which comparative morphometric studies of fasciolids in sympatric areas may be carried out making even the differentiation of potential intermediate forms possible. These new

Table 5 Significant differences detected when comparing *Fasciola hepatica* and *Fasciola gigantica* adult allometric curves in pairs of populations by ANCOVA test ($P<0.05$)

Pairs of variables	All three populations	Spain and Corsica	Spain and Burkina Faso	Corsica and Burkina Faso
tBW vs tBL	ETA: 12.6%, po: 1.0		ETA: 11%, po: 1.0	ETA: 12.8%, po: 1.0
tBWOv vs tBL			ETA: 2.5%, po: 0.5	
tOS_{max} vs tBL	ETA: 3.3%, po: 0.7			ETA: 4.0%, po: 0.7
tA-VS vs tBL	ETA: 8.1%, po: 1.0		ETA: 2.5%, po: 0.5	ETA: 9.5%, po: 1.0
tVS-Vit vs tBL	ETA: 10.9%, po: 1.0		ETA: 5.6%, po: 0.9	ETA: 7.9%, po: 1.0
tVit-P vs tBL	ETA: 4.5%, po: 0.9			ETA: 4.8%, po: 0.8
tVS-P vs tBL	ETA: 14.6%, po: 1.0		ETA: 4.8%, po: 0.8	ETA: 12.3%, po: 1.0
tTA vs tBL	ETA: 20.9%, po: 1.0		ETA: 11.3%, po: 1.0	ETA: 16.9%, po: 1.0
tTL vs tBL	ETA: 19.7%, po: 1.0		ETA: 9.1%, po: 1.0	ETA: 16.1%, po: 1.0
tTW vs tBL	ETA: 11.1%, po: 1.0		ETA: 8.0%, po: 1.0	ETA: 11.3%, po: 1.0
tTP vs tBL	ETA: 13.8%, po: 1.0		ETA: 7.2%, po: 0.9	ETA: 10.4%, po: 1.0
tBL vs tBA		ETA: 3.4%, po: 0.7		
tBW vs tBA	ETA: 34.8%, po: 1.0		ETA: 47.2%, po: 1.0	ETA: 31.2%, po: 1.0
tBWOv vs tBA	ETA: 11.2%, po: 1.0	ETA: 3.7%, po: 0.7	ETA: 13.8%, po: 1.0	ETA: 5.7%, po: 0.9
tBP vs tBA		ETA: 3.1%, po: 0.6		
tOS_{max} vs tBA				ETA: 2.9%, po: 0.6
tA-VS vs tBA	ETA: 5.2%, po: 1.0			ETA: 6.8%, po: 0.9
tVit-P vs tBA	ETA: 4.2%, po: 0.8			ETA: 4.3%, po: 0.8
tVS-P vs tBA	ETA: 2.7%, po: 0.6	ETA: 3.2%, po: 0.7		ETA: 2.8%, po: 0.6
tTA vs tBA	ETA: 23.4%, po: 1.0		ETA: 10.1%, po: 1.0	ETA: 20.5%, po: 1.0
tTL vs tBA	ETA: 9.1%, po: 1.0			ETA: 9.1%, po: 1.0
tTW vs tBA	ETA: 27.5%, po: 1.0	ETA: 2.7%, po: 0.6	ETA: 29.3%, po: 1.0	ETA: 27%, po: 1.0
tTP vs tBA	ETA: 12.1%, po: 1.0		ETA: 4.9%, po: 0.8	ETA: 10.1%, po: 1.0

For abbreviations, see Table 1
ETA Eta-squared statistics, *po* power

morphometric concepts provide appropriate tools for fasciolid adult stage and egg phenotyping as they are based on standardized measurements.

Adult size analysis

In relation to size, development has long been known to be a stable process (see Valero et al. 2005). There is a small number of models for growth laws that fit a large spectrum of magnitudes defined in morphological structures. Valero et al. (1996, 1998, 2001b) showed that the traditional morphometric measurements used for *F. hepatica* adults follow a logistic growth model with respect to time. This implies that the morphometric development of the *F. hepatica* adult is not limited but "damped" and does not exceed certain characteristic maximums.

In the *F. gigantica* allometries described, the maximum values (y_m) detected for most of the analysed measurements related to BL and the vertical growth gradient from the ventral sucker to the posterior end of the body are usually much higher than those detected in *F. hepatica*, especially Vit-P (see Table 1). Contrarily, *F. hepatica* showed higher maximum values (y_m) in those measurements related to BW and the horizontal growth gradient (BW, BWOv, A-VS and TW). These differences are related with the characteristic size of *F. gigantica* when compared to *F. hepatica*. Although the measurements anterior to the

Table 6 Comparative morphometric data of eggs in uterus from *Fasciola hepatica* and *Fasciola gigantica* populations from Spain, Corsica and Burkina Faso

Egg measurements (μm)	Spain, n=113	Corsica, n=101	Burkina Faso, n=142
Egg length, EL	107.30–152.70 129.80±0.83	100.22–155.62 126.13±1.14	129.61–204.51 156.80±1.07
Egg width, EW	52.44–89.11 69.59±0.60	54.99–87.72 72.32±0.66	61.63–112.56 89.45±0.75
Egg perimeter, EP	270.45–360.07 319.29±1.70	252.08–376.87 315.94±2.46	335.52–471.84 390.14±2.26
Egg roundness, ER	1.05–1.33 1.17±0.01	1.03–1.27 1.14±0.01	1.00–1.34 1.09±0.01
Egg area, EA	5,137.25–9,183.46 6,983.80±75.9	4,655.60–9,240.25 7,032.82±99.25	7,846.34–15,890.70 11,144.09±124.34
EL/EW ratio	1.46–2.54 1.88±0.02	1.39–2.12 1.75±0.02	1.32–2.64 1.77±0.02

All values are shown as range with mean±SE
SE Standard error, *n* sample size

ventral sucker do not seem to vary considerably between the species, the allometric study shows different morphological traits which make it possible to distinguish between *F. hepatica* and *F. gigantica* adults in the same host species. Despite the overlapping of most of the morphometric values of liver fluke adults from Africa and Europe, results obtained show 11 significant allometric differences in tBW vs tBL, tA-VS vs tBL, tVS-Vit vs tBL, tVS-P vs tBL, tTA vs tBL, tTL vs tBL, tTW vs tBL, tTP vs tBL, tBW vs tBA, tTA vs tBA, and tTP vs tBA.

Until now, authors have only considered morphometric characteristics without taking allometric growth into account, that is, using morphometric features whose values overlap in both fasciolids. The studies comparing both species of *Fasciola* have been undertaken in countries such as Iran, the Philippines, Thailand and Egypt where the two species coexist (Sahba et al. 1972; Kimura et al. 1984; Srimuzipo et al. 2000; Lotfy et al. 2002). In all of these studies, the specimens could not be clearly differentiated due to the overlap in the measurements obtained. All this is added to the complications of the existence of phenomena such as abnormal gametogenesis, diploidy, triploidy and mixoploidy, parthenogenesis or facultative gynogenesis and hybridization events (Itagaki et al. 1998; Terasaki et al. 2000; Fletcher et al. 2004). This comparative study of *F. hepatica* and *F. gigantica* adult specimens shows that all the values of classical measurements applied to liver fluke adults overlap in the two fasciolids, except VS-P which has proved to be a useful tool for the specific differentiation.

Adult shape analysis

Concerning shape, body roundness (BR) has shown to be a good potential species differentiation tool, as its measurements do not overlap between the two species and does not vary with age. Unfortunately, this parameter (BR) has never been used before. Other studies used ratios to characterize the two fasciolid species (Oshima et al. 1968; Akahane et al. 1970; Sahba et al. 1972; Kimura et al. 1984). Nevertheless, ratios may vary with age because of the different growth rates of the measurements involved. Therefore, they may not be useful for the specific differentiation. To ascertain which ratios could be useful as taxonomic tools, a study of the variation of each ratio with development was carried out. The only ratio that proved to be useful in the specific differentiation is BL/BW. It is also worth mentioning that even though the BAs of the species overlap, *F. hepatica* tends to be wider in size while *F. gigantica* tends to be much longer, thus, giving each species its characteristic body shape.

Egg size and shape analyses

Exhaustive studies on *F. hepatica* within the same endemic area, in which *F. gigantica* is not present, proved that egg measurements significantly differ between the different host species, and additional experimental studies showed that *F. hepatica* egg size of a given host isolate changed when experimentally transferred to a different host species (Valero et al. 2001a). Moreover, in areas sympatric for both liver fluke species, i.e. Asian countries, a large overlapping of egg measurements was detected (Watanabe 1962; Sahba et al. 1972; Kimura et al. 1984; Srimuzipo et al. 2000). The present study shows that although the morphometric values of liver fluke eggs somewhat overlap, three of them were significantly different between the two species: EL, EP and EA.

In conclusion, the quantification of the different size and shape of *F. hepatica* and *F. gigantica* gravid adults and eggs has been achieved in allopatric populations from Europe and Africa. The results obtained may be useful for the identification of the species of *Fasciola* present in countries where both species coexist.

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