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Liver fluke (*Fasciola hepatica*) naturally infecting introduced European brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk

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Abstract

Fascioliasis has recently been included in the WHO list of Neglected Zoonotic Diseases. Besides being a major veterinary health problem, fascioliasis has large underdeveloping effects on the human communities affected. Though scarcely considered in fascioliasis epidemiology, it is well recognized that both native and introduced wildlife species may play a significant role as reservoirs of the disease. The objectives are to study the morphological characteristics of *Fasciola hepatica* adults and eggs in a population of *Lepus europaeus*, to assess liver fluke prevalence, and to analyze the potential reservoir role of the European brown hare in northern Patagonia, Argentina, where fascioliasis is endemic. Measures of *F. hepatica* found in *L. europaeus* from northern Patagonia demonstrate that the liver fluke is able to fully develop in wild hares and to shed normal eggs through their faeces. Egg shedding to the environment is close to the lower limit obtained for pigs, a domestic animal whose epidemiological importance in endemic areas has already been highlighted. The former, combined with the high prevalence found (14.28%), suggest an even more important role in the transmission cycle than previously considered. The results obtained do not only remark the extraordinary plasticity and adaptability of this trematode species to different host species, but also highlight the role of the European brown hare, and other NIS, as reservoirs capable for parasite spillback to domestic and native cycle, representing a potentially important, but hitherto neglected, cause of disease emergence.

Keywords

Fasciola hepatica, *Lepus europaeus*, introduced species, reservoir

Introduction

Fascioliasis, traditionally considered as a veterinary health problem (Kaplan 2001), has recently been included in the World Health Organization list of Neglected Zoonotic Diseases (NZDs). This consideration is due to its emergence and re-emergence worldwide, affecting an estimated 17 million people (Mas-Coma *et al.* 2009), in a phenomenon which has partly been related to climate change (Mas-Coma *et al.* 2008; Afshan *et al.* 2014), and to the long-term pathogenic impact of this disease (Mas-Coma *et al.* 2014a). True human endemic areas have recently been described in which fascioliasis chronicity and superimposed repetitive infections pose pathological complications, indicating this disease to have large underdeveloping effects on the human communities affected (Valero *et al.* 2003, 2006a, 2008).

Fasciolid flukes follow a two-host life cycle, including a less specific adult stage which develops in many species of

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herbivorous mammals and even in a few omnivorous ones, and highly specific larval stages which only develop in given freshwater snail species of the family Lymnaeidae (Bargues and Mas-Coma 2005). With regard to the infection of animal reservoirs, the infectivity of the metacercarial infective stage from different animal species isolates has experimentally shown to be similar (Valero and Mas-Coma 2000; Valero *et al.* 2001a, 2011). Hence, the importance of ascertaining which animal species, including both domestic and sylvatic, develop a reservoir role in an endemic area.

Argentina presents a very widely distributed veterinary problem of fascioliasis in livestock (Olaechea 2007). Additionally, a recent analysis highlights that human fascioliasis in the country may have been overlooked in the past and its real epidemiological situation may currently be underestimated (Mera y Sierra *et al.* 2011). Surprisingly, geographical distribution of human infection does not fit that of fascioliasis in livestock, suggesting other transmission and epidemiological factors to be involved (Mera y Sierra *et al.* 2011).

Though scarcely considered, it is known that wildlife species may play a significant role as reservoirs of fascioliasis (Mas-Coma *et al.* 1988; Daszak *et al.* 2000; Bengis *et al.* 2004; Kruse *et al.* 2004; Polley 2005; Gayo *et al.* 2011; Mezo *et al.* 2013). Introduced non-indigenous species (NIS) are widely recognized as a source of disease (Daszak *et al.* 2000; Kelly *et al.* 2009). The importance and consequences of the introduction of NIS in fascioliasis has been the subject of several analyses, concerning both lymnaeids (Mas-Coma *et al.* 2003, 2005, 2009; Bargues and Mas-Coma 2005) and animal reservoirs (Mas-Coma *et al.* 2009).

Introduced into South America at the end of 19th century, the European brown hare (*Lepus europaeus*) represents one of the most widespread species of mammals (Bonino *et al.* 2010). The species has invaded almost all the extension of Argentina, Chile and Uruguay, and southern regions of Peru, Bolivia, Paraguay, and Brazil (Bonino *et al.* 2010). Despite old reports of *F. hepatica* in lagomorphs in general (Arru *et al.* 1967) and especifically infecting the hare in its original home range (Tropilo 1964; Kutzer and Frey 1976; Nickel and Gottwald 1979; Shimalov 2001; Ziege *et al.* 2009; Walker *et al.* 2011), the latter has been rarely considered in the epidemiology of the disease, particularly with regard to South American introduced populations. Additionally, phenotypic descriptions of adults and eggs of *F. hepatica* infecting natural populations of *L. europaeus* are lacking in the Neotropical region and even scarce worldwide.

The aims of the present article are to study the morphological characteristics of parasite adults and eggs in a population of *L. europaeus* in the northernmost part of Patagonia region (Argentina), to assess liver fluke prevalence and to analyze the potential reservoir role of this wild lagomorph in an area where fascioliasis is known to occur in livestock (Sidoti *et al.* 2009).

Materials and Methods

Host materials

Specimens of the European brown hare were obtained from the outskirts of Malargüe city (Mendoza province, Argentina), within the northernmost unit (Payunia district) of the Central Patagonia biogeographic province (Morrone 2006). Animals were captured by local hunters between August and September 2010, in an area of a mean altitude around 1500 m.a.s.l. A total of 35 refrigerated intestinal tracts and 27 livers were received for parasitological examination.

Parasitological techniques

Refrigerated intestinal tracts were immediately inspected for helminths, while faeces and livers were preserved in formaldehyde 4% for later examination. The content from the gas-

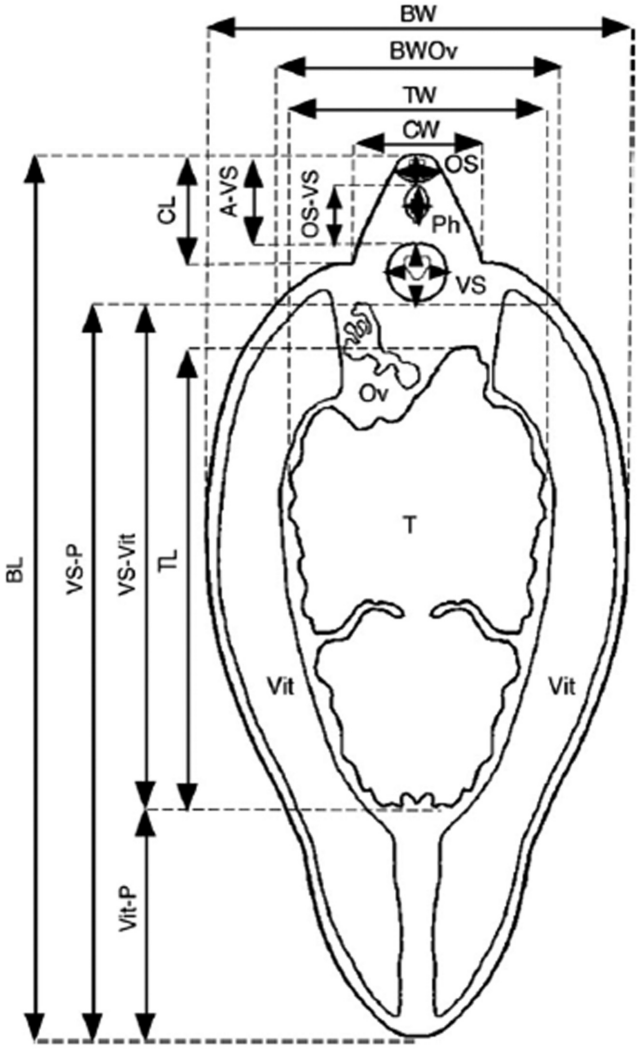

Fig. 1. Standardised measurements in gravid adults of *Fasciola hepatica*

Table I. Comparative morphometrics of 6 adult liver flukes in European brown hare (*Lepus europaeus*) (after Periago *et al.*, 2006). Data from liver flukes naturally obtained in domestic species by a) Valero *et al.* (2001a). Data from liver flukes experimentally obtained in Black rat (*Rattus rattus*) and Wistar rat (*Rattus norvegicus*) by a) Valero *et al.* (2001a), and b) Valero *et al.* (1998). All values are shown as range, with the mean and standard deviation (SD) in parentheses

	Natural infection				Experimental infection	
	European brown hare	Sheep[a]	Cattle[a]	Pig[a]	Wistar rat[a]	Black rat (50d.p.i.)[b]
Body length, BL	7.47–11.35 (9.13 ± 1.54)	4.90–31.11 (16.10 ± 4.80)	8.92–28.74 (19.07 ± 3.45)	10.03–24.87 (16.91 ± 3.00)	4.26–23.37 (14.24 ± 4.59)	5.7–13.1 (9.4)
Body width, BW	3.23–4.53 (4.01 ± 0.47)	1.58–12.55 (7.11 ± 2.27)	3.16–12.95 (8.39 ± 1.51)	5.21–11.45 (8.50 ± 1.55)	1.90–12.95 (8.11 ± 3.08)	2.0–5.1 (3.5)
BW at ovary level, BWOv	2.18–3.52 (2.94 ± 0.58)	–	–	–	–	–
Body perimeter, BP	19.64–24.79 (22.37 ± 2.09)	12.39–68.35 (43.13 ± 12.54)	23.60–67.73 (52.03 ± 8.33)	30.23–66.39 (47.32 ± 7.79)	12.28–62.91 (40.33 ± 13.12)	–
Body roundness, BR	1.35–2.19 (1.70 ±0.34)	–	–	–	–	–
Cone length, CL	0.85–1.49 (1.06 ± 0.26)	0.80–3.04 (2.06 ± 0.31)	1.38–3.06 (2.11 ± 0.31)	1.60–3.38 (2.33 ± 0.33)	0.85–3.08 (1.95 ± 0.42)	1.1–2.3 (1.7)
Cone width, CW	1.23–2.32 (1.59 ± 0.39)	1.20–3.90 (2.46 ± 0.43)	1.48–3.75 (2.76 ± 0.41)	2.03–4.47 (3.18 ± 0.47)	1.03–3.73 (2.60 ± 0.72)	–
Maximum diameter of the oral sucker, OSmax	0.61–0.69 (0.63 ± 0.04)	0.37–1.06 (0.72 ± 0.10)	0.54–0.92 (0.73 ± 0.07)	0.57–1.15 (0.84 ± 0.10)	0.32–0.86 (0.63 ± 0.14)	–
Minimum diameter of the oral sucker, OSmin	0.500.56 (0.52 ± .02)	–	–	–	–	–
Maximum diameter of the ventral sucker, VSmax	0.32–0.49 (0.41 ± 0.07)	0.43–1.25 (0.94 ± 0.14)	0.69–1.88 (1.02 ± 0.11)	0.75–1.29 (1.07 ± 0.10)	0.40–1.20 (0.88 ± 0.20)	0.5–0.8 (0.6)
Minimum diameter of the ventral sucker, VSmin	0.17–0.24 (0.20 ± 0.03)	–	–	–	–	–
Distance between the anterior end of the and the ventral sucker, A-VS	0.65–1.23 (0.99 ± 0.24)	0.79–3.35 (2.11 ± 0.36)	1.49–2.98 (2.27 ± 0.28)	1.62–3.35 (2.36 ± 0.35)	0.90–2.80 (1.97 ± 0.45)	1.1–2.1 (1.6)
Distance between the oral sucker and the ventral sucker, OS-VS	0.33–0.80 (0.65 ± 0.24)	0.06–2.56 (1.51 ± 0.31)	0.86–2.29 (1.63 ± 0.27)	1.03–2.55 (1.70 ± 0.33)	0.60–2.26 (1.51 ± 0.39)	0.6–1.4 (1.0
Distance between the ventral sucker and the union of the vitelline glands, VS-Vit	4.22–6.55 (5.69 ± 1.05)	–	–	–	–	–
Distance between the union of the vitelline glands and the posterior end of the body, Vit-P	1.29–2.74 (2.17 ± 0.55)	–	–	–	–	–
Distance between the ventral sucker and the posterior end of the body, VS-P	6.73–9.26 (7.86 ± 0.94)	3.16–27.39 (13.03 ± 4.45)	6.63–24.95 (15.81 ± 3.19)	7.58–20.18 (13.52 ± 2.64)	2.76–19.58 (11.41 ± 4.03)	4.2–9.8 (7.0)

Pharynx length, PhL	0.48–0.68 (0.55 ± 0.12)	–	–	–	–	–
Pharynx width, PhW	0.30–0.32 (0.31 ± 0.01)	–	–	–	–	–
Testicular length, TL	2.85–4.08 (3.56 ± 0.57)	–	–	–	–	–
Testicular width, TW	1.872.72 (2.09 ± 0.36)	–	–	–	–	–
Testicular perimeter, TP	12.16–7.96 (10.84 ± 1.69)	–	–	–	–	–
Body area, BA	22.7–32.4 (25.7 ± 3.9)	6.08–216.77 (84.70 ± 46.96)	19.06–196.35 (114.31 ± 34.25)	42.47–182.03 (101.69 ± 32.65)	6.90–191.79 (89.76 ± 50.03)	–
Oral sucker area, OSA	0.27–0.32 (0.3 ± 0.02)	0.08–0.66 (0.34 ± 0.09)	0.18–0.57 (0.37 ± 0.07)	0.22–0.68 (0.43 ± 0.09)	0.07–0.39 (0.25 ± 0.09)	
Ventral sucker area, VSA	0.6–0.7 (0.6 ± 0.05)	0.15–1.23 (0.71 ± 0.19)	0.37–1.70 (0.80 ± 0.15)	0.45–1.25 (0.88 ± 0.17)	0.12–1.06 (0.63 ± 0.26)	–
Pharynx area, PhA	0.11–0.16 (0.13 ± 0.03)	–	–	–	–	–
Testicular area, TA	5.57–6.78 (6.24 ± 0.49)	–	–	–	–	–
BL/BW ratio	1.68–3.22 (2.33 ± 0.63)	1.33–4.17 (2.33 ± 0.44)	1.40–3.48 (2.30 ± 0.37)	1.50–2.68 (2.02 ± 0.27)	1.18–2.99 (1.85 ± 0.36)	–
BWOv/CW ratio	1.39–2.79 (1.92 ± 0.52)	–	–	–	–	–
OSA/VSA ratio	0.43–0.57 (0.50 ± 0.05)	0.25–0.70 (0.49 ± 0.08)	0.22–0.74 (0.47 ± 0.08)	0.30–0.70 (0.49 ± 0.08)	0.19–0.71 (0.42 ± 0.09)	–
BL/VS-P ratio	1.32–1.07 (1.16 ± 0.09)	1.13–1.55 1.25 ± 0.06	1.00–1.36 1.21 ± 0.04	1.18–1.37 1.26 ± 0.03	1.06–1.60 1.27 ± 0.08	–

50d.p.i., 50 days post-infection

Table II. Measurements taken from 280 eggs of *Fasciola hepatica*, recovered from faeces of European brown hare (*Lepus europaeus*). All values are shown as range, with the mean and standard deviation (SD) in parentheses. EL, egg length (μm); EW, egg width (μm); EA, egg area (μm^2), ER, egg ratio; n.i., natural infection; e.i., experimental infection. Data from rodents and other domestic species by a) Valero *et al*. (2002), and b) Valero *et al*. (2001a)

Host	Geographical location	EL	EW	EA	ER
Brown Hare (*Lepus europaeus*), n.i.	Northern Patagonia, Argentina	90.5–143.7 (120.0 ± 8.9)	56.6–86.2 (68.9 ± 4.9)	6142.4–11408.7 (8275.1 ± 919.3)	1.3–2.3 (1.7 ± 0.2)
Mouse (*Mus musculus*), n.i.[a]	Corsica island, Mediterranean Sea	117–122 (119 ± 2)	60–83 (74 ± 7)	7158–9887 (8836 ± 809)	–
Black rat (*Rattus rattus*), n.i.[a]	Corsica island, Mediterranean Sea	122–148 (133 ± 8)	60–74 (67 ± 3)	7148–10344 (9011 ± 685)	–
Cattle (*Bos Taurus*), n.i.[a]	Corsica island, Mediterranean Sea	125–149 (136 ± 9)	68–83 (74 ± 6)	9128–11300 (10114 ± 801)	–
Cattle (*Bos Taurus*), n.i.[b]	Northern Bolivian Altiplano	105.3–155.9 (132.0 ± 10.5)	61.7–82.5 (71.1 ± 4.4)	5286.5–9676.8 (7170.2 ± 802.5)	1.6–2.3 (1.9 ± 0.2)
Sheep (*Ovis aries*), n.i.[b]	Northern Bolivian Altiplano	114.8–151.2 (130.8 ± 7.1)	65.5–81.4 (72.6 ± 3.9)	5998.2–8608.5 (7238.0 ± 532.8)	1.5–2.2 (1.8 ± 0.1)
Pig (*Sus scrofa domestica*), n.i.[b]	Northern Bolivian Altiplano	73.8–148.6 (123.8 ± 11.3)	58.1–82.5 (71.8 ± 4.4)	3988.7–8626.9 (6836.0 ± 820.4)	1.1–2.1 (1.7 ± 0.2)
Donkey (*Equus asinus*), n.i.[b]	Northern Bolivian Altiplano	96.4–140.8 (125.4 ± 8.3)	63.3–84.7 (75.0 ± 3.7)	5562.6–8686.2 (7177.4 ± 646.1)	1.3–2.0 (1.7 ± 0.1)
Wistar rat (*Rattus norvegicus*), e.i.[a]	Corsica island, Mediterranean Sea	122–148 (134 ± 6)	63–80 (70 ± 4)	7681–11841 9376 ± 866)	–
Wistar rat (*Rattus norvegicus*), e.i.[b]	Northern Bolivian Altiplano	98.1–144.2 (124.6 ± 7.8)	56.9–80.8 (67.6 ± 3.4)	4836.2–7982.3 (6380.1 ± 510.8)	1.4–2.2 (1.9 ± 0.2)

trointestinal tracts from each hare were thoroughly examined following standard methods (Egerton *et al.* 1979).

Previously preserved faecal samples were analyzed by means of two methods: Sheather's sucrose flotation technique (MacPherson and McQueen 1993) and Lumbreras' rapid sedimentation technique (Lumbreras *et al.* 1962). Sediment obtained from Lumbrera's technique was subsequently passed through a 140 μm sieve. Both techniques were performed with three grams of material. Slides from Sheather's technique and filtered sediment from Lumbrera's technique were microscopically examined. Faecal counts (eggs per gram = epg; oocysts per gram = opg) were determined in every sample.

Liver fluke adults were recovered from preserved livers, while eggs were concentrated by means of sedimentation and filtration from the remaining faecal material previously found 'positive'. Adult worms were stained with Grenacher's borax carmine and mounted in Canada balsam between slide and coverglass but without coverglass pressure (Valero *et al.* 2005, 2012).

Measurement techniques and data analysis

Egg characteristics studied were length (EL) and width (EW) in μm. The product of these 2 dimensions was used as a measure of egg size (EL × EW = ES μm^2), and the ratio as a measure of shape (EL/EW = ER) (Poulin 1997; Abrous *et al.* 1998; Valero *et al.* 1998, 2001a, 2002). For egg classification, egg size was considered according to recent updates on this characteristic (Valero *et al*. 2009; Mas-Coma *et al.* 2014b), and by taking into account the influence of the host species (Valero *et al.* 1998, 2001a, 2002),

For adult fasciolids, the following standardized measurements were taken (Valero *et al.* 2005; Periago *et al.* 2006) (Fig. 1): (i) lineal biometric characters (mm): 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 oral sucker (OS max), minimum diameter of oral sucker (OS min), maximum diameter of ventral sucker (VS max), minimum diameter of ventral sucker (VS min), distance between the anterior end of the body and ventral sucker (A–VS), distance between the oral sucker and ventral sucker (OS–VS), distance between the oral 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) length (TL), testicular space width (TW), testicular space perimeter (TP); (ii) areas (mm^2): body area (BA), oral sucker area (OSA), ventral sucker area (VSA), pharynx area (PhA), testicular space area (taking both testes together, TA); (iii) 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).

Morphometric measurements used for *F. hepatica* adults follow a logistic growth model with respect to time (Valero *et al.* 2001a,b, 2005). This implies that the morphometric development of the fasciolid adult is not limited but 'damped' and does not exceed certain characteristic maximum (Valero *et al.* 1998, 2006b). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table I).

Results

Five faecal samples were detected positive to *Fasciola hepatica* (14.28%, 2.7–25.8% CI 0.95), while 33 showed *Eimeria* sp. oocysts (94.28%, 86.61–100% CI 0.95). No nematode and cestode eggs or adults were observed. Faecal counts showed between 1 and 3 epg for liver fluke (mean 2.08 epg, ± 1.25), and 91.73 mean opg (± 155.84) for *Eimeria* sp.

Twenty-two liver fluke adults were recovered from a single liver, but only six of them could be measured (Table I), while a total of 280 eggs were recovered from faeces and measured (Table II).

Discussion

Each trematode species has its own adult and egg phenotype, generally within a specific range (Valero *et al.* 2009). However, small host body mass offers limited microhabitat (e.g. liver) and places a physical constraint upon the trematode body size and number of flukes that can fit in (Poulin 1997; Valero *et al.* 2001a, 2005); while it has been associated with diminished egg size (Valero *et al.* 2002). Consequently, the final host species decisively influences the size of adults and eggs of *F. hepatica* (Valero *et al.* 2001a,b, 2005, 2009).

Measures of *F. hepatica* found in *L. europaeus* from Malargüe department proved to be among the smaller described in adults and eggs recovered from naturally and experimentally infected murid rodents, lagomorphs and domestic species (see Tables I and II) (Abrous *et al.* 1998; Valero *et al.* 1998, 2001a, 2002). With regard to adult liver flukes, it shall be considered that samples were preserved in formaldehyde 4% during, at least, two months, which might have slightly decreased measures. Size of the fasciolids from the European brown hare appears similar to fasciolids of 50 days of age experimentally obtained in the Black rat (Valero *et al.* 1998) (Table I). However, the size of *F. hepatica* eggs found in faecal samples of the hares fully overlap not only with those of natural and experimental infections in murid rodents, but also with those of natural infections in cattle and other domestic animals (Valero *et al.* 2001a, 2002). All in all, the data obtained indicates that the liver fluke is able to fully develop in wild hares and to shed normal eggs through their faeces.

Additionally, the heavy parasite burden observed (22 liver flukes in a single liver) and the small adult size described strongly suggest an effect of crowding, a phenomenon reflected in a decreased adult development when the number of flukes is high (Valero *et al.* 2006b). Meanwhile, due to experimental evidence of a direct relation between uterus size and the numbers of eggs shed per gram of faeces (Valero *et al.* 2001b, 2011), the reduced uterus development as consequence of smaller adults (Poulin 1997) may explain the low epg observed.

Although *F. hepatica* infection in wild *L. europaeus* has been detected before in its original European range, to the best of our knowledge only one report deals with that aspect in South America (Kleinman *et al.* 2004). Unfortunately, the information provided is only restricted to the local prevalence found. The high prevalence found in our study (14.28%, 2.7–25.8% CI 0.95) strongly contrasts with the very low one registered (<1‰) in the aforementioned study (Kleiman *et al.* 2004). Our results suggest an even more important role in the transmission cycle than previously considered, at least in given areas.

Considering a daily defaecation rate of 410 faecal pellets per hare (Novaro *et al.* 1992), a pellet weight between 1 and 1.4 gr (Kleiman *et al.* 2004), and the epg here obtained, each hare could shed to the environment a daily rate of 410–1,722 eggs of *F. hepatica*. This result is close to the lower limit obtained for pigs (2,000–195,000 eggs/individual/day), a domestic animal whose epidemiological importance in endemic areas has already been highlighted (Mas-Coma *et al.* 1997, 2005).

Parasites tend to have threshold levels of host populations size below which they are unable to persist (Tompkins and Poulin 2006). The population dynamics of the European brown hare, as a competent host for liver fluke (i.e. hosts in which the parasites can develop normally), may allow parasite spillback by amplifying the total number of infective stages and increasing the infection burdens in populations of other susceptible hosts (native or domestic) (Kelly *et al.* 2009; Poulin *et al.* 2011). This situation set the stage for the European brown hare, a NIS, to alter local parasite dynamics in ways that could lead to disease emergence and an outbreak (Rachowicz *et al.* 2005; Thieltges *et al.* 2009; Poulin *et al.* 2011).

The results obtained do not only remark the extraordinary plasticity and adaptability of this trematode species to different host species, but also highlight the role of the European brown hare, and other NIS, as reservoirs capable for parasite spillback to domestic and native cycle, representing a potentially important, but hitherto neglected, cause of disease emergence. The present finding of *F. hepatica* in hares indicates that the geography of the populations of this lagomorph will be in need to be considered when analysing the distribution and extent of fascioliasis infection risk areas (Fuentes *et al.* 1999, 2001; Afshan *et al.* 2014) in Argentina and also in other South American endemic countries where the European hare has been introduced.

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