Research Paper

Author for correspondence:
M.A. Valero,
E-mail: madela.valero@uv.es

CAMBRIDGE
UNIVERSITY PRESS

Variability of *Calodium hepaticum* eggs from sigmodontine host species through geometric morphometric analysis

M.C. Fantozzi[1,2] , M. Reguera-Gomez[1] , P.M. Beldomenico[2] ,
S. Mas-Coma[1,3] , M.D. Bargues[1,3] and M.A. Valero[1,3]

[1]Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain; [2]Laboratorio de Ecología de Enfermedades, Instituto de Ciencias Veterinarias del Litoral (ICIVET LITORAL), Universidad Nacional del Litoral- CONICET, Esperanza, Santa Fe, Argentina and [3]Consorcio Centro de Investigación Biomédica en Red (CIBER), Área de Enfermedades Infecciosas (CIBERINFEC), ISC III, Madrid, Spain

Abstract

Calodium hepaticum is a zoonotic nematode with a worldwide distribution. Although the host range of *C. hepaticum* includes a wide spectrum of mammals (including humans), this parasite is predominantly associated with the families Muridae and Cricetidae. Several Sigmodontinae species from Argentina were found to be infected by *C. hepaticum*, with a high prevalence in *Akodon azarae*. The present study focuses on *C. hepaticum* eggs from natural infection of three species of sigmodontine rodents from Argentina. Eggs were genetically characterized (intergenic 18S rRNA region). The objectives of this work are: (i) to propose a new analytical methodology; and (ii) to morphologically characterize *C. hepaticum* eggs, from three Sigmodontinae species (*A. azarae*, *Calomys callidus* and *Oligoryzomys flavescens*). Analyses were made by the Computer Image Analysis System based on the new standardized measurements and geometric morphometric tools. The resulting factor maps clearly illustrate global size differences in the parasite eggs from the three Sigmodontinae species analysed. The degree of similarity between egg populations was assessed through pairwise Mahalanobis distances, showing that the largest distances were detected between parasite eggs from *C. callidus* and *O. flavescens*. Herein, the phenotypical plasticity of *C. hepaticum* eggs is shown. Significant positive correlations were obtained between each egg parasite principal component 1 and rodent corporal characteristics: weight; liver weight; rodent length; and rodent body condition. The usefulness of the geometric morphometric analysis in studies of the relationship between *C. hepaticum* and its host must be highlighted. The high prevalence observed in *A. azarae*, associated with the wide size range of the parasite eggs evidenced by principal component analysis, suggests *A. azarae* to be the Sigmodontinae host species that plays the most important role as reservoir host for *C. hepaticum* in the New World.

Introduction

Calodium hepaticum Moravec 1982 (Trichinellida: Capillaridae) (syn. *Capillaria hepatica*) is a zoonotic nematode with a worldwide distribution (Schmidt, 2001; Fuehrer, 2014a,b). Although the host range of *C. hepaticum* includes a wide spectrum of mammals (including humans) (Fuehrer *et al.*, 2011; Fuehrer, 2014a,b), this parasite is predominantly associated with the families Muridae (Murinae, Deomyinae) and Cricetidae (Arvicolinae, Neotominae, Cricetinae, Sigmodontinae, Gerbilinae and Cricetomyinae) (Schmidt, 2001; Fuehrer, 2014a). Of these rodents, the Norway rat *Rattus norvegicus*, Berkenhout, 1769, seems to be the most important host species worldwide, with high prevalence reported in several continents (Duque *et al.*, 2012; Moreira *et al.*, 2013; Fuehrer, 2014a), followed by the black rat *Rattus rattus*, Linnaeus, 1758 (Almeida-Silva *et al.*, 2011; Moreira *et al.*, 2013), and the house mouse *Mus musculus*, Linnaeus, 1758 (Gotardo *et al.*, 2000; Fuehrer, 2014a).

In the New World, the predominant Muroidea rodents are Sigmodontinae (Cricetidae), comprising approximately 84 genera and nearly 380 species (Patton *et al.*, 2015). Sigmodontine rodents are predominantly distributed in South America with about 85 endemic genera (D'Elía & Pardiñas, 2015). At least 108 species of sigmodontine rodents have been recorded in Argentina, including representatives of nine tribes and some *incertae sedis* taxa (Patton *et al.*, 2015; Teta *et al.*, 2018; Formoso & Teta, 2019).

The potential role that Sigmodontinae play as hosts of *C. hepaticum* has been little investigated, but a recent report showed that they might be important for parasite dynamics, at least in South America (Fantozzi *et al.*, 2018). Several Sigmodontinae species from Argentina were found to be infected by *C. hepaticum*, with high prevalence in *Akodon azarae* Fischer, 1829 (Fantozzi *et al.*, 2018).

Adults of *C. hepaticum* are found in the liver. Hepatic infections occur following ingestion of embryonated eggs by a mammal. After hatching, the larvae penetrate through the intestinal wall, migrate into the hepatic portal system, and develop as adults in the hepatic parenchyma (Spratt & Singleton, 2001), where gravid females lay immature eggs which are then exposed to the next host after the death and decomposition of the infected host or when it becomes prey (Spratt & Singleton, 2001; Camargo *et al.*, 2010). Moreover, it has been suggested that cannibalism may be important in the transmission among rats (Rothenburger *et al.*, 2014).

Several examination techniques are available to detect the presence of *C. hepaticum* eggs. The most widely used approach for the *C. hepaticum* infection has been based on morphology, morphometry and histopathology (Ferreira & Andrade, 1993; Andrade & Andrade, 2004; Almeida *et al.*, 2012; Duque *et al.*, 2012; Fantozzi *et al.*, 2019). In turn, a novel procedure for taxonomic species identification through morphological and morphometric characteristics of parasite eggs in capillariids has recently been proposed (Borba *et al.*, 2021b).

The aetiological diagnosis of helminth infections is usually based on the finding of characteristic eggs, larvae, or proglottids in biological samples. Most of the helminths living in the intestinal tract or in associated organs produce eggs. Each helminth species produces eggs that display intraspecific variation in size/shape, but which are mostly highly uniform in their morphological phenotype, and size and shape are therefore used as criteria for egg identification of biological samples, that is, the shape and size of helminth eggs are characteristic features of most helminth species, being commonly used as diagnostic criteria. Furthermore, eggshells are usually smooth and may vary considerably in thickness, depending on the helminth species (Ash & Orihel, 2010). Thus, various shell structure particularities, such as the mucoid polar opercula present in *Trichuris*, *Calodium* or *Capillaria*, are also important in identification. Concretely, eggs of *Trichuris* species are associated with prominent mucoid polar opercula and *Capillaria* and *Calodium* species eggs are associated with inconspicuous mucoid polar opercula. The subjectivity of the terms such as prominent or inconspicuous highlights the need to use markers that allow this characteristic to be quantified.

Adults of *C. hepaticum* are found in the liver and are identified through ultrasound, biopsy or necropsy. Only in spurious cases, have the eggs been observed in faeces (coproanalysis). In those cases, eggs are identified by means of morphology and morphometry. Classical morphometric techniques are, unfortunately, not able to characterize the features of the egg mucoid polar opercula, let alone assert host species influence on the parasite egg. For this reason, new tools should be applied to advance in diagnostics. Furthermore, morphological analyses are necessary to complement molecular techniques (Valero *et al.*, 2001, 2009, 2012; García-Sánchez *et al.*, 2019, 2020; Reguera-Gomez *et al.*, 2021).

Regarding the morphometric characterization of Trichinellida representatives, geometric morphometrics has proven to be a useful tool to differentiate male adults (García-Sánchez *et al.*, 2019) and eggs (García-Sánchez *et al.*, 2020) of *Trichuris* spp. populations infecting non-human primates and *Sus scrofa domestica* that can complement molecular data. Nevertheless, hitherto, no studies of this kind have been carried out in representatives of other genera such as *Calodium*. Furthermore, these new morphometric concepts provide adequate tools to characterize the morphological phenotype of *Calodium* eggs to assess the influence of different factors, such as the host species.

The objectives of this work are: (i) to propose a new analytical methodology; and (ii) to characterize morphologically eggs of *C. hepaticum* from natural infection of three species of sigmodontine rodents from Argentina (*A. azarae* Fischer, 1829, *Calomys callidus* Thomas, 1916 and *Oligoryzomys flavescens* Waterhouse, 1837). To this end, *C. hepaticum* eggs from adult parasites that had previously been genetically characterized were used.

Material and methods

Study sites

The study area is located in central Argentina, in the Espinal Ecoregion, characterized by a warm and humid climate (Matteucci, 2018). There are four well marked seasons (delimited by solstices and equinoxes), with abundant rains in autumn and summer (annual precipitation of 900 to 1200 mm) and with an average annual temperature of 18°C (Matteucci, 2018).

Sigmodontine rodents were captured in riparian environments. The localities were Esperanza (ES), Santa Fe Province (31° 24′ 20″ S–60° 58′ 16″ W) and La Picada (LP), Entre Ríos Province (31° 44′ 06″ S–60° 21′ 53″ W), about 80 km one from the other (fig. 1). Both sites are dominated by grasslands and situated in close proximity to important water courses that are tributaries of the Paraná River basin. ES is located on the El Salado River, and LP is located on the Las Conchas stream.

Sample collection

Rodents were captured between June 2014 and September 2016, in three-night trapping sessions carried out every five weeks. Eight trapping sites were selected (four fixed grids in ES and four in LP) consisting of 0.5 ha where 50 Sherman-type live-traps were placed and baited with pelletized commercial dog food. Plots were located in places with natural grassland. Traps were checked every morning and trapped rodents were transported to a field laboratory, anaesthetized by inhalation of isoflurane and sacrificed by cervical dislocation. The following data were taken and recorded from each individual: weight (g); total length (head + body + tail (cm)); length hind foot (mm); ear length (mm); and sex. Body condition was evaluated by estimating through palpation the degree of fat cover over the vertebral column and the pelvic bones, giving a score between 2 and 10, following Burthe *et al.* (2006). Rodents were identified at species level by assessing their cranial morphology (Massoia & Fornes, 1965; Massoia, 1973; Goncálvez & De Oliveira, 2004; Udrizar Sauthier *et al.*, 2020), according to Patton *et al.* (2015) for rodent taxonomy.

Four hundred and nine individual Sigmodontinae livers were weighed and examined, immediately after the hosts had been euthanized, under a magnifying lens to find evidence of nematodes. A total of 64 (15.6%) livers were found to be infected, recovering in all cases female nematodes. Eggs of *C. hepaticum* were obtained from liver disintegration.

This study has its limitations. Previous studies of *Fasciola hepatica* egg size showed characteristic morphometric traits to be related to their definitive host species (Valero *et al.*, 2002, 2009). Nevertheless, this phenomenon has not been previously described in the species of the genus *Calodium*. *Calodium hepaticum* eggs used in this work were recovered from wild Sigmontinidae rodents from Argentina and not from experimental host samples. The prevalence of infected rodents in the geographical area of study has been previously cited by the
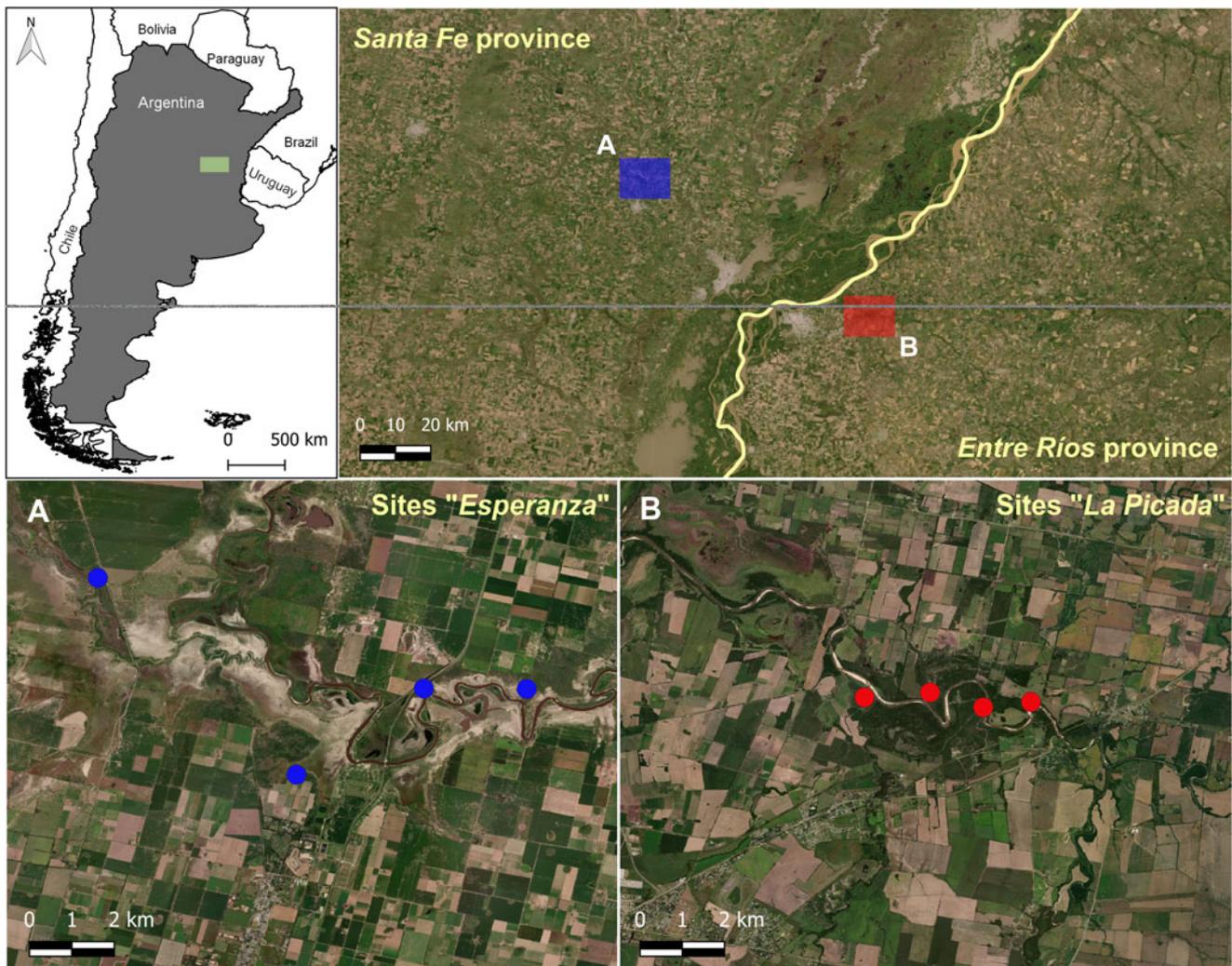
Fig. 1. Localities studied in the El Espinal ecoregion, Argentina: (A) Esperanza in Santa Fe Province; and (B) La Picada in Entre Ríos Province.

authors, detecting *C. hepaticum* infection in 64 of 409 rodents (15.6%) (Fantozzi *et al.*, 2018). The role of the main reservoir or secondary reservoir of a helminth species is marked by the prevalence of infection. Consequently, the low prevalence present in *C. callidus* and *O. flavescens* makes it difficult to plan a study design with an adequate number of samples.

Molecular analysis of C. hepaticum *adults from liver samples*

Genomic DNA from individual worms was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer) according to the manufacturer's protocol. Genomic DNA concentration and purity were assessed using the SPECTROstar Nano and the MARS Data Analysis Software (BMGLabtech, Germany). The 18S rRNA partial gene was amplified by polymerase chain reaction (PCR), using primers 18S 965 and 18S 1573R (Powers *et al.*, 2009). The PCR was performed in an Ivema T-18 thermocycler (Ivema Desarrollos, Argentina) using 5 µl of 5× Phire® reaction buffer, 200 µM dNTP, 0.4 pM of each primer, 100 ng of genomic DNA, and 0.5 µl of Phire® Hot Start II DNA polymerase (Finnzymes, Finland) and completed to 25 µl with molecular grade water. PCR conditions were as follows: 1 cycle at 98°C for 3 min, followed by 35 cycles at 98°C for 10 s, 57°C for 20 s,

72°C for 35 s and 1 final cycle at 72°C for 5 min (Fantozzi *et al.*, 2018). A negative control was included in each amplification run. Homologies were performed using the Basic Local Alignment Search Tooln program from the United States National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

Phenotypic analyses: proposal for a new analytical methodology

Measurement techniques

A total of 314 eggs of *C. hepaticum* were analysed (218 eggs from 11 individuals of *A. azarae*, 57 eggs from three individuals of *C. callidus* and 39 eggs from two individuals of *O. flavescens*). Initially, each egg was isolated with a micropipette, thoroughly washed with distilled water and immediately placed between microscope slides and coverslips with a water-based mounting medium to improve their image and preservation.

With the image analysis software, lineal biometric characters, areas and ratios of the eggs were obtained. Only well-preserved eggs were used for measurements which were made with a microscope (Nikon Eclipse E 800) at 100× magnification and using image analysis software (ImagePro Plus, version 5.1 for

Fig. 2. Eggs of *Calodium hepaticum* in different sigmodontine hosts: (A) *Akodon azarae*; (B) *Calomys callidus*; (C) *Oligoryzomys flavescens* (optical microscopy, original magnification = ×400). Scale bars: 20 μm.

Windows, Media cybernetics, Silver Spring, USA) for images captured by a digital camera (Nikon Coolpix 5400). Representative images of the photographed eggs are shown in fig. 2.

Nineteen measurements were used. To identify the ones that give some valuable information about the egg morphology and were, therefore, suitable for principal component analysis (PCA), an iterative process of measurement combinations was performed.

Measurements were specifically assayed in this study due to the lack of standardized parameters. These measurements consider the characteristic shape of these eggs, with their two mucoid polar opercula of different size. In order to properly describe each of the two independently, the one of the larger size is denominated A, while the smaller one is denominated B.

Apical width mucoid polar opercula (L1 in A, L5 in B). Width measured from the top of the mucoid polar opercula.

Medium width mucoid polar opercula (L2 in A, L6 in B). Width measured at the middle of the mucoid polar opercula.

Basal width mucoid polar opercula (L3 in A, L7 in B). Measurements made inside the eggshell. Egg basal width mucoid polar opercula.

Length mucoid polar opercula (L4 in A, L8 in B). Length measured between the top and the end of the mucoid polar opercula.

Wall thickness at its midpoint (L9). Wall thickness width measured at the central axis of the egg.

Egg length excluding mucoid polar opercula length (L10). Measurements made inside the eggshell. Egg length without considering the mucoid polar opercula length.

In addition, other measurements classically considered in other helminth eggs were also applied:

Egg area (EA). Area included in the figure defining the egg outline.

Egg perimeter (EP). Length of the egg outline.

Egg roundness (ER). Defined as (perimeter^2/4π × area). 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 (Valero *et al.*, 2009, 2018).

Egg length (EL). Feret diameter along major axis of egg.

Egg width (EW = L11). Feret diameter along minor axis of egg.

Size ratio (SR). Size length over size width (EL/EW).

Other measurements obtained from each egg were available in the options of the image analysis software:

Diameter (maximum). The maximum segment that passes through the centre and joins two opposite points of a circle.

Diameter (minimum). The minimum segment that passes through the centre and joins two opposite points of a circle.

Diameter (mean). The mean between minimum and maximum diameter.

Numerical methods

Biometrical data were examined using multivariate analysis. Current statistical techniques in morphometrics make it possible to test the null hypothesis of conspecific populations being simply the allometric extension of each other, provided that a common allometric trend is identifiable (Rohlf & Marcus, 1993). Morphological differences were quantified by geometric morphometrics, which includes validated methods for analysing variation in organismal shape (Klingenberg, 1996). These procedures focus on the retention of geometric information throughout a study and provide efficient, statistically powerful analyses that can readily relate abstract, multivariate results to the physical structure of the original specimens (Slice, 2007).

The PCA is an estimator of certain parametric structure characteristics of the population. It may also be used as a dimension-reduction technique that identifies orthogonal linear combinations of the original variables that most efficiently reproduce sample variability. The latter use is particularly important in morphometric research as the number of shape variables to be analysed can be very large and often exceeds reasonable sample sizes (Slice, 2007). For the analysis, we built a two-dimensional morphospace plot based on the first two principal components.

A way of correcting the problem of the existence of variation in size due to ontogeny or other causes would be to remove statistically the effect size present within the sample of each group and then apply canonical discriminant analysis to the samples already

corrected for within-group differences in size. Thus, size-free canonical discriminant analysis was used on the covariance matrix of measurements transformed into logarithms to assess morphometric variation among samples. This technique consists of removing the effect of within-group ontogenetic variation, regressing each character separately on the within group first principal component (a multivariate size estimator) (Periago *et al.*, 2008; Valero *et al.*, 2018).

The resulting 'allometry-free' (effect of size on morphological variation), or size-free, variables were submitted to a canonical variate analysis (CVA) and Mahalanobis distances were derived (Mahalanobis, 1936). The Mahalanobis distance is a statistical technique that can be used to measure how distant a point is from the centre of a multivariate normal distribution, that is, it measures the separation of two groups. Mahalanobis distances were used to quantify shape divergence between groups. In the present analysis, the degree of shape divergence between *C. hepaticum* eggs populations was assessed through pairwise Mahalanobis distances (Ashrafi *et al.*, 2015; García-Sánchez *et al.*, 2020).

The PCA, CVA and Mahalanobis distances were performed using both the Collecting Landmarks for Identification and Characterization package and the available online XYOM software (https://xyom.io/) and tested by non-parametric permutation tests with 1000 iterations each. Values were considered statistically significant when $P < 0.05$ (Dujardin & Dujardin, 2019; García-Sánchez *et al.*, 2019; Reguera-Gomez *et al.*, 2021). Nevertheless, to avoid matrix singularities, some measurements were discarded because of their partial overlap with other measurements.

The following seven non-redundant measurements (i.e. one is not included in another) were considered in the egg analysis: L2 medium width mucoid polar opercula width A; L4 length mucoid polar opercula A; L6 medium width mucoid polar opercula B; L8 length mucoid polar opercula B; L9 wall thickness at its midpoint and L10 egg length excluding mucoid polar opercula length; and L11 egg width (see fig. 3).

Statistical analyses

Statistical analyses were conducted using the R version 4.0.0 software (The R Project for Statistical Computing; http://www.rproject.org). Bivariant correlations (Spearman's rank correlation) were calculated for body host variables (body weight, liver weight, rodent length and rodent body condition) and egg size (principal component 1 (PC1)). Also, generalized linear mixed models (GLMM) were calculated. All models included host identification as a random intercept to account for the lack of independence of observations. PC1 was used as response variable and host species (= host sample size effect), and body condition and liver weight were used as independent variables. Season and year of capture were also included in the models to control for potential confounding. The 'glmer' function of the lme4 Package was used. The relevance of the terms was evaluated with the second order Akaike information criterion (AICc) to account for small sample sizes (Arnold, 2010; Burnham *et al.*, 2011). When the inclusion of each term did not reduce AICc values in two or more units (ΔAICc < 2), it was dropped from the model. Results were considered statistically significant when $P < 0.05$.

To evaluate the possible influence of geographical location on the morphological phenotype of parasite eggs, a non-parametric test (Spearman correlation test) between the Mahalanobis
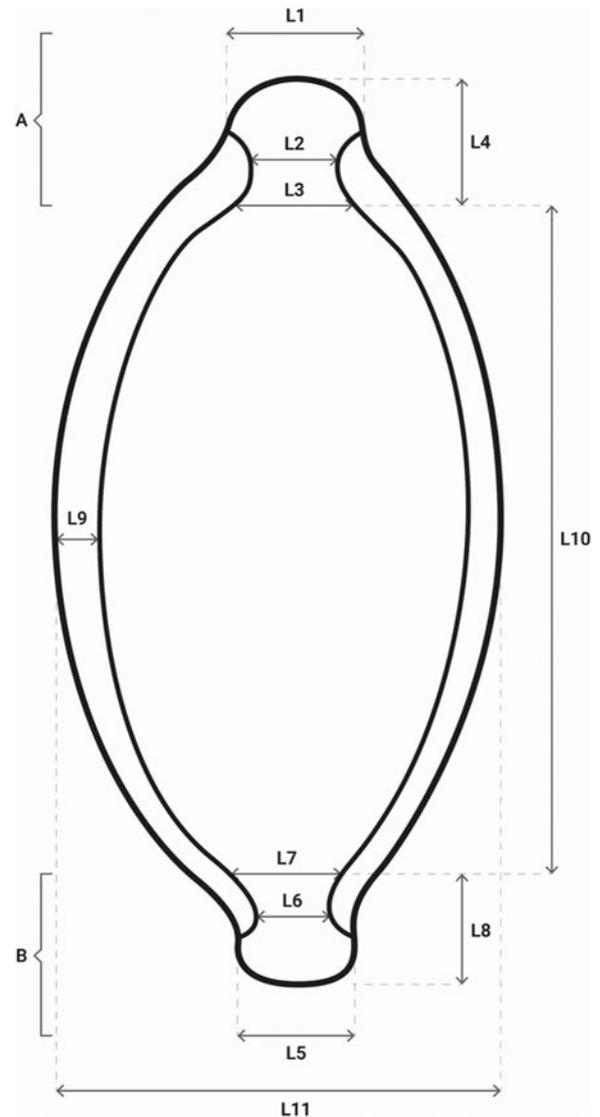

Fig. 3. Standardized measurements for the morphometric phenotyping of *Calodium hepaticum* eggs in sigmodontine species. The mucoid polar opercula larger in size is denominated A, while the smaller one is denominated B. L1 apical width mucoid polar opercula A; L2 medium width mucoid polar opercula A; L3 basal width mucoid polar opercula A; L4 length mucoid polar opercula A; L5 apical width mucoid polar opercula B; L6 medium width mucoid polar opercula B; L7 basal width mucoid polar opercula B; L8 length mucoid polar opercula B; L9 wall thickness at its midpoint; L10 egg length excluding mucoid polar opercula length; and L11 egg width.

distances and geographical altitudinal/latitudinal and geographical distances (km) was applied.

Results

Molecular criteria for species identification

The resulting sequences (excluding primers) were 100% identical (600/600) to the corresponding 18S rRNA sequence of *C. hepaticum* obtained from *R. norvegicus* (KT875351) and from *A. azarae* and *C. callidus* (MG686613) in Argentina (Fantozzi *et al.*, 2018). Complete identity was also observed with 18S rRNA sequences of *C. hepaticum* in the Old World from *Arvicola terrestris* (JX456635) (Guardone *et al.*, 2013), *R. norvegicus* (KT875351) and *A. amphibious* (JX456635-JX456636) in Switzerland (Bolukbas *et al.*, 2020).

Table 1. Comparative morphometric data (extreme values, mean and standard deviation) of *Calodium hepaticum* eggs from three Sigmodontinae rodent species (*Akodon azarae*, *Calomys callidus* and *Oligorizomys flavescens*).

Egg measurements (μm)	A. azarae (n = 218)	C. callidus (n = 57)	O. flavescens (n = 39)
apical width mucoid polar opercula A (L1)	4.96–12.00 9.68 ± 1.03	5.01–9.350 7.62 ± 0.82	8.43–10.53 9.52 ± 0.55
medium width mucoid polar opercula A (L2)	3.93–9.06 6.86 ± 0.93	4.07–9.26 6.61 ± 1.02	5.32–8.49 6.75 ± 0.70
basal width mucoid polar opercula A (L3)	6.05–11.85 9.02 ± 0.97	5.20–10.95 8.63 ± 1.00	7.67–10.69 8.91 ± 0.80
length mucoid polar opercula A (L4)	2.92–7.91 5.85 ± 0.94	3.03–6.60 4.45 ± 0.72	4.30–7.87 5.81 ± 0.81
apical width mucoid polar opercula B (L5)	4.83–10.91 8.84 ± 1.10	4.04–8.65 6.87 ± 0.92	7.08–9.88 8.48 ± 0.67
medium width mucoid polar opercula B (L6)	3.04–8.92 5.98 ± 0.82	3.25–8.07 6.14 ± 0.93	4.96–7.66 6.13 ± 0.65
basal width mucoid polar opercula B (L7)	5.96–11.43 8.564 ± 0.86	5.20 –9.94 8.16 ± 0.95	6.43–10.56 8.49 ± 0.99
length mucoid polar opercula B (L8)	2.88–7.32 5.29 ± 0.92	2.18–6.42 3.99 ± 0.84	3.21–7.62 5.34 ± 0.87
wall thickness at its midpoint (L9)	0.93–4.21 2.26 ± 0.69	1.78–4.06 2.88 ± 0.41	1.41–3.33 2.27 ± 0.38
egg length excluding mucoid polar opercula length (L10)	49.24–70.69 61.42 ± 3.86	49.03–67.19 56.14 ± 3.83	53.50–66.79 60.18 ± 3.86
area (EA)	1063.09–2323.96 1662.07 ± 266.79	1265.51–2413.24 1600.80 ± 216.32	1180.06–1915.50 1572.85 ± 170.54
perimeter (EP)	135.67–195.87 170.20 ± 10.95	139.04–194.06 159.04 ± 9.72	148.20–183.59 166.52 ± 8.45
roundness (ER)	1.19–1.65 1.40 ± 0.09	1.17–1.38 1.26 ± 0.04	1.26–1.58 1.40 ± 0.07
length (EL)	52.81–76.34 66.99 ± 4.19	51.64–71.26 60.36 ± 3.84	58.12–72.26 65.76 ± 4.27
width (EW = L11)	24.29–42.22 33.66 ± 4.02	29.14–44.82 34.26 ± 3.56	27.85–37.52 32.75 ± 2.48
size ratio (SR)	1.61–2.55 2.01 ± 0.21	1.44–2.13 1.77 ± 0.16	1.73–2.51 2.01 ± 0.19
diameter (maximum)	52.68–75. 37 66.86 ± 4.16	51.75–71.28 60.33 ± 3.82	57.84–72.11 65.57 ± 4.13
diameter (minimum)	24.50–42.29 33.80 ± 4.23	28.96–44.81 34.15 ± 3.58	28.23–37.38 32.76 ± 2.47
diameter (mean)	40.00–58.83 50.33 ± 3.63	40.90–58.05 47.24 ± 3.19	43.37–53.71 49.16 ± 2.54

n = number of eggs.

Morphological characterization of C. hepaticum *eggs from natural infection of three sigmodontine rodent species by means of the new analytical proposal*

The overall size of *C. hepaticum* eggs obtained from geometric morphometric analysis samples is shown in table 1.

The comparison of measurements of *C. hepaticum* eggs coming from three Sigmodontinae rodent species by GLMM analysis is shown in table 2, presenting significant differences between eggs from *C. callidus* and *A. azarae*.

Also, the study of egg phenotypical characteristics was carried out by PCA, where PC1 and principal component 2 together, contributed 76% of the total variation. The resulting factor maps (fig. 4) clearly illustrate global size differences in the parasite eggs from the three Sigmodontinae species analysed and illustrate three areas corresponding to: (a) subgroup covering *A. azarae* and *O. flavescens* eggs; (b) subgroup covering *A. azarae*, *C. callidus* and *O. flavescens* eggs; and (c) subgroup covering *A. azarae* and *C. callidus* eggs (fig. 4). Interestingly, the results show that *C. hepaticum* eggs in *A. azarae* and *O. flavescens* share larger size values and that smaller specimens are present mainly in *A. azarae* and *C. callidus*.

The degree of similarity between egg populations was assessed through pairwise Mahalanobis distances. These distances were calculated comparing eggs from three host species with each other. Malahanobis distances are displayed in table 3. When comparing *C. hepaticum* eggs from *A. azarae* with respect to eggs from

Table 2. Significant differences in measurements between *Calodium hepaticum* eggs coming from three Sigmodontinae rodent species (*Akodon azarae*, *Calomys callidus* and *Oligorizomys flavescens*) by generalized linear mixed models analysis (P < 0.05).

	A. azarae	C. callidus
A. azarae		
C. callidus	diameter (maximum), EL, EP, ER, SR, L1, L4, L5, L8, L10	
O. flavescens	–	–

All models included host identification as a random intercept to account for the lack of independence of observations.

C. callidus, and eggs from *A. azarae* with respect to eggs from *O. flavescens*; the detected distances were smaller than in the comparison of *C. hepaticum* eggs from *C. callidus* with respect to eggs from *O. flavescens*. Summing up, the largest distances were detected between parasite eggs from *C. callidus* and *O. flavescens*.

Host body variables and the intraspecific variability of parasite eggs

Significant correlations were obtained between each egg parasite PC1 ($n = 314$) and rodent corporal characteristics: weight (rho: 0.322, $P < 0.001$); liver weight (rho: 0.214, $P < 0.001$); rodent length (rho: 0.352, $P < 0.001$); and rodent body condition (rho: 0.310, $P < 0.001$). Summing up, positive correlations between egg parasite PC1 and host variables were detected.

The final GLMM showed *C. callidus* associated with PC1. Smaller *C. hepaticum* eggs are present mainly in *C. callidus* (table 4).

Finally, there was no consistent correlation between the size-free pattern of variation and altitudinal/latitudinal and geographical distances (km). No significant values between Mahalanobis distances and geographical distances were detected (data not shown).

Discussion

According to previous *C. hepaticum* egg measurements described from *R. rattus*, length averaged 60 μm (range 40 μm to 75 μm) and 37 μm (range 27–41 μm) in width (Choe *et al.*, 1993; Fugassa *et al.*, 2008; Carvalho-Costa *et al.*, 2009; Resendes *et al.*, 2009; Buńkowska-Gawlik *et al.*, 2017). The average length of the *C. hepaticum* eggs analysed herein (66.99 μm, range: 52.81–76.34 μm, in *A. azarae*; 60.36 μm, range 51.64–71.26 μm, in *C. callidus*, 65.76 μm, range 58.12–72.26 μm, in *O. flavescens*) (table 1) falls within this range although our minimum record is slightly smaller in *C. callidus*. The maximum width (44.82 μm) detected in the *C. hepaticum* eggs from *C. callidus* analysed in the present study is also remarkably larger than previously reported values (range 27–41 μm) (Choe *et al.*, 1993; Fugassa *et al.*, 2008; Carvalho-Costa *et al.*, 2009; Resendes *et al.*, 2009; Gonçalves *et al.*, 2012; Buńkowska-Gawlik *et al.*, 2017). Summing up, previous descriptions showed a wide range in the measurements included, indicating the necessity of studies analysing the influence of the different factors involved such as geographical origin or definitive host influence.

Morphological characterization of *C. hepaticum* eggs from natural infection of three species of sigmodontine rodents applying the new analytical proposal

Our study provides the first geometric analysis of the egg morphometry of *C. hepaticum* from sigmodontine rodents from Argentina, PCA proving to be an efficient method to analyse *C. hepaticum* eggs. The measurements chosen to be included in the PCA have been proposed for the first time. Through the centroids obtained in PCA, parasite eggs from *C. callidus*, were differentiated from those from *A. azarae* and *O. flavescens*. *Calodium hepaticum* eggs from *C. callidus* appeared separated from *A. azarae* and *O. flavescens*. On the other hand, the factor map shows an overlapping area between the parasite eggs originating from the three rodent species. The subgroups observed in fig. 4 are likely to reflect ecological or geographical influences.
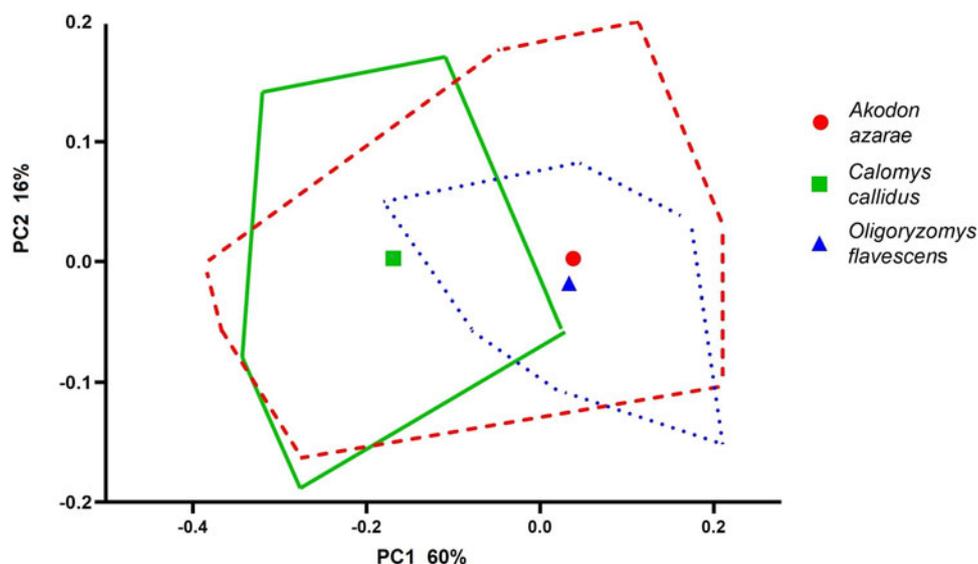

Fig. 4. Factor map corresponding to individual principal component analysis attributed to each egg of *Calodium hepaticum* derived from different sigmodontine host species: *Akodon azarae*; *Calomys callidus*; and *Oligoryzomys flavescens*. Each sample is projected onto the first (PC1, 60%) and second (PC2, 16%) principal components. The horizontal axis is PC1, the vertical axis is PC2. Together, they contributed 76% to the total variation. Each group is represented by its perimeter. Circle, square and triangle represent the centroid in each group.

Table 3. Mahalanobis distances between the three *Calodium hepaticum* egg groups according to the three host species: *Akodon azarae*; *Calomys callidus*; and *Oligoryzomys flavescens*.

	A. azarae	*C. callidus*	*O. flavescens*
A. azarae	0.00		
C. callidus	**2.16***	0.00	
O. flavescens	0.19	**2.26***	0.00

*statistically different over 1000 permutations with Bonferroni correction ($P < 0.05$).

In previous studies, the influence of the geographical location or host species on the size of helminth adults and eggs has been reported, for example, in species of *Fasciola* (Valero *et al.*, 2001, 2002, 2009, 2012, 2018; Ashrafi *et al.*, 2015) or *Trichuris* (Garcia-Sánchez *et al.*, 2019, 2020). Nevertheless, these phenomena have so far not been described in the genus *Calodium*.

With respect to representatives of Nematoda eggs, previous analyses using geometric morphometry focused only on *Trichuris* species. García-Sánchez *et al.* (2020) used a total of eight lineal egg measurements (maximum width of polar opercula, minimum width of polar opercula, base width of polar opercula, length of polar opercula measured from exterior midpoint to the narrow midpoint, total length of polar opercula measured from the exterior midpoint to the base midpoint, wall thickness at its midpoint, wall thickness in contact with polar opercula and interior length of the egg) in the characterization of the size of *Trichuris* eggs. The study of interspecific variability enables the analysis of the morphological patterns in different *Trichuris* sp. eggs associated with different host species [(macaque (*Macaca sylvanus*), eastern black-and-white colobus (*Colobus guereza kikuyuensis*), grivets (*Chlorocebus aethiops*) and Brazza's monkey (*Cercopithecus neglectus*)]. In contrast to that study, herein seven measurements are used: L2 medium width mucoid polar opercula A; L4 length mucoid polar opercula A; L6 medium width mucoid polar opercula B; L8 length mucoid polar opercula B; L9 wall thickness at its midpoint; L10 egg length excluding mucoid polar opercula length; and L11 egg width. The different measurements applied, compared with the proposal by García-Sánchez *et al.* (2020), are based on the fact that herein the two mucoid polar opercula are characterized independent from each other. It is noteworthy that the asymmetry detected in *Calodium* eggs in polar opercula is more pronounced than that detected in polar opercula of *Trichuris* eggs.

The most frequently morphological parameters reported by other researchers are length and width (among others: Carvalho-Costa *et al.*, 2009; Almeida *et al.*, 2012; Macchioni *et al.*, 2013; Klisiowicz *et al.*, 2014; Sinniah *et al.*, 2014; Buńkowska-Gawlik *et al.*, 2017). Several reports on Capillaridae eggs detected distinct morphotypes, for example, in *Trichuris* sp. (Petržeľková *et al.*, 2010; Klaus *et al.*, 2017; Garcia-Sánchez *et al.*,

2020; Kamani *et al.*, 2021), or species of the genera *Aonchotheca*, *Baruscapillaria*, *Capillaria*, *Calodium*, *Echinocoleus*, *Eucoleus*, *Pearsonema* and *Tridentocapillaria* (Borba, 2019; Borba *et al.*, 2021a). Interestingly, *C. hepaticum* eggs from *Meriones persicus* and *R. rattus* are described as having a 'very peculiar morphology', that is, a punctuated and a radial eggshell ornamentation, with a thick eggshell (5.54 μm) (Borba *et al.*, 2021b). Furthermore, Borba *et al.* (2021b) recently reported a new methodology for Capillariid species identification based on artificial intelligence technology. In this case, despite having taken the measurements of the two mucoid polar opercula, the discriminant analysis between representatives of Capillariidae was carried out using only the length and width of the eggs as measurements (Borba *et al.*, 2021b).

Host body variables and intraspecific variability of parasite eggs

The capacity of organisms to present different phenotypes under varying environmental conditions (phenotypical plasticity) has been studied since the Neo-Darwinian synthesis (Pigliucci, 2005). However, few studies have quantitatively assessed the influence that the host body characteristics exercises on egg morphology of helminth parasites. In some helminth species, previous studies show that the host animal body can strongly influence the phenotype of the egg stage. As pointed out by Poulin (1997), the host mass correlates with the space available for parasites in various organs, which may put physical constraints upon helminth body size. Thus, in the case of *F. hepatica*, Valero *et al.* (2002) showed that the small host body mass of rats and mice was associated with diminished *F. hepatica* egg size.

The present study focuses on the intraspecific variability of *C. hepaticum* eggs associated with the host body characteristics. The PC1 of the egg samples can be interpreted as a measure of overall egg size, and positive correlations were detected between eggs PC1 and host rodent corporal characteristics (weight, liver weight, rodent length and rodent body condition). Furthermore, GLMM corroborate the association between egg PC1 and host variables. Herein, the phenotypical plasticity of *C. hepaticum* eggs related to host body characteristics is shown. Future research with an adequate sample size is needed to define this parasite–host relationship.

Fantozzi *et al.* (2018) documented the occurrence of *C. hepaticum* in two rodent assemblages from central Argentina, basing the diagnosis on morphological characteristics, histopathological examination and 18S ribosomal RNA sequences, showing a prevalence of 41.2% in *A. azarae*, 13.8% in *C. callidus* and 7.1% in *O. flavescens*. The high prevalence observed in *A. azarae*, associated with the wide size range of the parasite eggs evidenced by PCA, suggests *A. azarae* to be the Sigmodontinae host species that plays the most important role as reservoir host for *C. hepaticum* in the New World.

Table 4. Generalized linear mixed models showing the association between principal component 1 (PC1) and host variables.

PC1 ~ body condition + host species + liver weight + year + season + (1 | ID)	Estimate	Standard error	*P* value	AICc*
Intercept	0.07543	0.02011	0.00280	
C. callidus	**−0.17389**	**0.03731**	**0.00055**	**548.65**
O. flavescens	0.04118	0.04403	0.36821	549.48

The significant coefficients are shown in boldface type ($P < 0.05$). *Akaike information criterion (AICc) value increments if the variable is dropped.

The present study proposes a new standardized approach able to characterize egg morphological features of *C. hepaticum* eggs. As a matter of fact, without having previously demonstrated this possibility, the results obtained in the present work are expected to lay the groundwork for descriptive or comparative morphometric studies. In the latter case, this tool allows the evaluation of the possible influence of the different factors on the morphological phenotype of parasite eggs such as the host species influence, geographical location or spurious infections in humans, among others.

References

Almeida-Silva MJF, Fava CD, Potenza M, Reis F and Campos ADC (2011) Diagnosis of *Capillaria hepatica* in *Rattus rattus* by histopathology. pp. 233–235. In *7th International conference on urban pests*. Ouro Preto, Brazil.

Almeida F, Caldas R, Corrêa C, Rodrigues-Silva R, Siqueira N and Machado-Silva JR (2012) Co-infections of the cestode *Echinococcus vogeli* and the nematode *Calodium hepaticum* in the hystricomorphic rodent *Agouti paca* from a forest reserve in Acre, Brazil. *Journal of Helminthology* **87**(4), 489–493.

Andrade SB and Andrade ZA (2004) Experimental hepatic fibrosis due to *Capillaria hepatica* infection (differential features presented by rats and mice). *Memorias del Instituto Oswaldo Cruz* **99**(4), 399–406.

Arnold TW (2010) Uninformative parameters and model selection using Akaike's information criterion. *Journal of Wildlife Management* **74**(6), 1175–1178.

Ash LR and Orihel TC (2010) *Atlas de parasitología humana* [Atlas of human parasitology]. 5th edn. 540 pp. Editorial Médica Panamericana, Buenos Aires, Bogotá, Caracas, Madrid, México, Porto Alegre. [In Spanish.]

Ashrafi K, Valero MA, Peixoto RV, Artigas P, Panova M and Mas-Coma S (2015) Distribution of *Fasciola hepatica* and *F. gigantica* in the endemic area of Guilan, Iran: relationships between zonal overlap and phenotypic traits. *Infection, Genetics and Evolution* **31**(1), 95–109.

Bolukbas CS, Demirtas S, Gurler AT, Inal S, Acici M and Umur S (2020) Molecular characterization of *Calodium hepaticum* in grey dwarf hamster (*Cricetulus migratorius*). *Parasitology International* **78**, 102133.

Borba VT (2019) *Taxonomia integrativa*, Filogenia e paleodistribuição de capilarídeos [Integrative taxonomy, phylogeny and palaeodistribution of *Filogenia*]. PhD thesis, Universidade do Estado do Rio de Janeiro, Rio de Janeiro. [In Portuguese].

Borba V, Enoki M, Lopes-Torres EJ, Machado-Silva JR and Iñiguez AM (2021a) New data on eggshell structure of capillariid species: a SEM perspective. *Parasitology Research* **120**(4), 963–970.

Borba VH, Martin C, Machado-Silva JR, Xavier SCC, de Mello FL and Iñiguez AM (2021b) Machine learning approach to support taxonomic species discrimination based on helminth collections data. *Parasites & Vectors* **14**(1), 230.

Buńkowska-Gawlik K, Perec-Matysiak A, Burzyńska K and Hildebrand J (2017) The molecular identification of *Calodium hepaticum* in the wild brown rat (*Rattus norvegicus*) in Poland. *Acta Parasitologica* **62**(4), 728–732.

Burnham KP, Anderson DR and Huyvaert KP (2011) AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behavioral Ecology and Sociobiology* **65**(1), 23–35.

Burthe S, Telfer S, Lambin X, Bennett M, Carslake D, Smith A and Begon M (2006) Cowpox virus infection in natural field vole *Microtus agrestis* populations: delayed density dependence and individual risk. *Journal of Animal Ecology* **75**(6), 1416–1425.

Camargo LMA, Camargo JDSA, de Souza Vera LJ, Barreto PDTC, Tourinho EK and de Souza MM (2010) Capillariasis (Trichurida, Trichinellidae, *Capillaria hepatica*) in the Brazilian Amazon: low pathogenicity, low infectivity and a novel mode of transmission. *Parasites & Vectors* **3** (1), 11.

Carvalho-Costa FA, Silva AG, de Souza AH, *et al.* (2009) Pseudoparasitism by *Calodium hepaticum* (syn. *Capillaria hepatica*; *Hepaticola hepatica*) in the Negro River, Brazilian Amazon. *Transactions of the Royal Society of Tropical Medicine & Hygiene* **103**(10), 1071–1073.

Choe G, Lee HS, Seo JK, Chai JY, Lee SH, Eom KS and Chi JG (1993) Hepatic capillariasis: first case report in the Republic of Korea. *American Journal of Tropical Medicine and Hygiene* **48**(5), 610–625.

D'Elía G and Pardiñas UFJ (2015) Subfamily Sigmodontinae Wagner, 1843. pp. 63–70. In Patton JL, Pardiñas UFJ, and D'Elía G (Eds) *Mammals of South America. Volume 2 – rodents*. Chicago, IL, University of Chicago Press.

Dujardin S and Dujardin JP (2019) Geometric morphometrics in the cloud. *Infection, Genetics and Evolution* **70**(1), 189–196.

Duque BA, Aranzazu D, Agudelo-Florez P, London AF, Quiroz VH and Rodas JD (2012) *Rattus norvegicus* as an indicator of circulation of *Capillaria hepatica* and *Taenia taeniaeformis* on a groceries trade center of Medellín, Colombia. *Biomédica* **32**(4), 510–518.

Fantozzi MC, Robles MR, Beldomenico PM and Monje LD (2018) *Calodium hepaticum* (Nematoda: Capillariidae) in wild rodent populations from Argentina. *Parasitology Research* **117**(9), 2921–2926.

Fantozzi MC, Sanchez A, Ciorciari AS, Peña FE, Canal A and Beldomenico PM (2019) *Calodium hepaticum* (Trichuroidea: Capillariidae) infection in *Akodon azarae*: first description of histopathological lesions in sigmodontinae. *Journal of Parasitology* **105**(1), 75–78.

Ferreira LA and Andrade ZA (1993) *Capillaria hepatica*: a cause of septal fibrosis of the liver. *Memórias do Instituto Oswaldo Cruz* **88**(3), 441–447.

Formoso AE and Teta PV (2019) Richness, endemism and conservation of sigmodontine rodents in Argentina. *Mastozoología Neotropical* **26**(1), 99–116.

Fuehrer HP (2014a) An overview of the host spectrum and distribution of *Calodium hepaticum* (syn. *Capillaria hepatica*): Part 1—Muroidea. *Parasitology Research* **113**(2), 619–640.

Fuehrer HP (2014b) An overview of the host spectrum and distribution of *Calodium hepaticum* (syn. *Capillaria hepatica*): Part 2—mammalia (excluding Muroidea). *Parasitology Research* **113**(2), 641–651.

Fuehrer HP, Igel P and Auer H (2011) *Capillaria hepatica* in man—an overview of hepatic capillariosis and spurious infections. *Parasitology Research* **109**(4), 969–979.

Fugassa MH, Taglioretti V, Gonçalves ML, Araújo A, Sardella NH and Denegri GM (2008) *Capillaria* spp. eggs in Patagonian archaeological sites: statistical analysis of morphometric data. *Memórias do Instituto Oswaldo Cruz* **103**(1), 104–105.

García-Sánchez AM, Rivero J, Callejón R, Zurita A, Reguera-Gomez M, Valero MA and Cutillas C (2019) Differentiation of *Trichuris* species using a morphometric approach. *International Journal of Parasitology: Parasites and Wildlife* **9**(1), 218–223.

García-Sánchez AM, Reguera-Gomez M, Valero MA and Cutillas C (2020) Differentiation of *Trichuris* species eggs from non-human primates by geometric morphometric analysis. *International Journal of Parasitology: Parasites and Wildlife* **12**(2), 214–219.

Gonçalves AQ, Ascaso C, Santos I, Serra PT, Juliao GR and Orlandi PP (2012) *Calodium hepaticum*: household clustering transmission and the finding of a source of human spurious infection in a community of the Amazon region. *Plos Neglected Tropical Diseases* **6**(12), e1943.

Goncálvez PR and De Oliveira JA (2004) Morphological and genetic variation between two sympatric forms of *Oxymycterus* (Rodentia: Sigmodontinae): An evaluation of hypotheses of differentiation within the genus. *Journal of Mammalogy* **85**(1), 148–161.

Gotardo BM, Andrade RG and Andrade ZA (2000) Hepatic pathology in *Capillaria hepatica* infected mice. *Revista da Sociedade Brasileira de Medicina Tropical* **34**(4), 341–346.

Guardone L, Deplazes P, Macchioni F, Mag JM and Mathis A (2013) Ribosomal and mitochondrial DNA analysis of Trichuridae nematodes of carnivores and small mammals. *Veterinary Parasitology* **197**(1–2), 364–369.

Kamani J, Massetti L, Olubade T, Balami JA, Samdi KM, Traub RJ, Colella V and González-Miguel J (2021) Canine gastrointestinal parasites as a potential source of zoonotic infections in Nigeria: a nationwide survey. *Preventive Veterinary Medicine* **192**(1), 105385.

Klaus A, Zimmermann E, Röper KM, Radespiel U, Nathan S, Goossens B and Strube C (2017) Co-infection patterns of intestinal parasites in arboreal primates (proboscis monkeys, *Nasalis larvatus*) in Borneo. *International Journal of Parasitology: Parasites and Wildlife* **6**(3), 320–329.

Klingenberg CP (1996) Multivariate allometry. pp. 23–49. In Marcus LF, Corti M, Loy GJP, Naylor A and Slice D (Eds) *Advances in Morphometrics, Proceedings of the 1993 NATO–ASI on Morphometrics, NATO ASI, Ser. A, Life Sciences*. Plenum Publishers, New York.

Klisiowicz DDR, Reifur L, Shimada MK, Haidamak J, Cognialli RCR and Ferreira T (2014) High occurrence of *Calodium hepaticum* (syn. *Capillaria hepatica*) spurious infection in a village in the Atlantic Forest of southern Brazil. *Memórias do Instituto Oswaldo Cruz* **109**(3), 371–373.

Macchioni F, Chelucci L, Guardone L, Mignone W, Prati MC and Magi M (2013) *Calodium hepaticum* (Nematoda: Capillaridae) in a red fox (*Vulpes vulpes*) in Italy with scanning electron microscopy of the eggs. *Folia Parasitologica* **60**(2), 102–104.

Mahalanobis PC (1936) On the generalized distance in statistics. *Proceedings of the National Institute of Science of India* **2**(1), 49–55.

Massoia E (1973) Descripción de *Oryzomys fornesi*, nueva especie y nuevos datos sobre algunas especies y subespecies argentinas del subgénero *Oryzomys* (*Oligoryzomys*) (Mammalia – Rodentia – Cricetidae) [Description of

Oryzomys fornesi, a new species and new data on some Argentine species and subspecies of the subgenus *Oryzomys* (*Oligoryzomys*) (Mammalia – Rodentia – Cricetidae)]. *Revista de Investigaciones Agropecuarias Serie 1 Biologia yProduccion Animal* **110**, 21–38. [In Spanish.]

Massoia E and Fornes A (1965). Contribución al conocimiento de los roedores miomorfos argentinos vinculados con la fiebre hemorrágica Argentina [Contribution to the knowledge of Argentine myomorphic rodents linked to Argentine hemorrhagic fever]. Ministerio de Asuntos Sociales y Salud Pública de la Nación, Difusión de la Comisión Nacional Coordinadora para el Estudio y Lucha contra la Fiebre Hemorrágica Argentina. [In Spanish.]

Matteucci SD (2018) Ecorregión Espinal [Spinal Ecoregion]. pp. 349–390. In Morello J, Matteucci SD, Rodriguez AF and Silva ME (Eds) *Ecorregiones y complejos ecosistémicos Argentinos* [Argentine ecoregions and ecosystemic complexes]. 2nd ed. Buenos Aires. Fallecido 8/2013. [In Spanish.]

Moreira VL, Giese EG, Silva DC, Melo FT, Furtado AP, Maldonado A Jr and Santos JN (2013) *Calodium hepaticum* (Nematoda: Capillariidae) in synanthropic rodents (*Rattus norvegicus* and *Rattus rattus*) in Eastern Amazonia. *Revista Brasileira de Parasitología Veterinaria* **22**(2), 265–269.

Patton JL, Pardiñas UF and D'Elía G (2015) *Mammals of South America, volume 2: rodents*. 1336 pp. Chicago, IL, University of Chicago Press.

Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, Desquesnes M, Curtale F and Mas-Coma S (2008) First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infection, Genetics and Evolution* **8**(1), 51–58.

Petrželková KJ, Hasegawa H, Appleton CC, Huffman MA, Archer CE, Moscovice LR, Mapua MI, Singh J and Kaur T (2010) Gastrointestinal parasites of the chimpanzee population introduced onto Rubondo Island National Park, Tanzania. *American Journal of Primatology* **72**(4), 307–316.

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology & Evolution* **20**(9), 481–486.

Poulin R (1997) Egg production in adult trematodes: adaptation or constraint? *Parasitology* **114**(2), 195–204.

Powers TO, Neher DA, Mullin P, Esquivel A, Giblin-Davis RM, Kanzaki N, Stock SP, Mora MM and Uribe-Lorio L (2009) Tropical nematode diversity: vertical stratification of nematode communities in a Costa Rican humid lowland rainforest. *Molecular Ecology* **18**(5), 985–996.

Reguera-Gomez M, Valero MA, Oliver-Chiva MC, *et al.* (2021) First morphogenetic analysis of parasite eggs from *Schistosomiasis haematobium* infected sub-Saharan migrants in Spain and proposal for a new standardised study methodology. *Acta Tropica* **223**(1), 106075.

Resendes AR, Amaral AF, Rodrigues AFS and Almeria S (2009) Prevalence of *Calodium hepaticum* (Syn. *Capillaria hepatica*) in house mice (*Mus musculus*) in the Azores archipelago. *Veterinary Parasitology* **160**(3–4), 340–343.

Rohlf FJ and Marcus LF (1993) A revolution in morphometrics. *Trends in Ecology & Evolution* **8**(4), 129–132.

Rothenburger J, Himsworth CG, Chang V, LeJeune M and Leighton FA (2014) *Capillaria hepatica* in wild Norway rats (*Rattus norvegicus*) from Vancouver, Canada. *Journal of Wildlife Diseases* **50**(3), 628–633.

Schmidt S (2001) *Investigation on the occurrence of* Capillaria hepatica *and metacestodes of Cyclophillida in wild mice in Germany*. 145 pp. PhD thesis, Institute of Parasitology, Faculty of Veterinary Medicine, University of Leipzig, Germany.

Sinniah B, Narasiman M, Habib S and Gaik Bei O (2014) Prevalence of *Calodium hepaticum* and *Cysticercus fasciolaris* in urban rats and their histopathological reaction in the livers. *Journal of Veterinary Medicine* **14**(1), 172829.

Slice DE (2007) Geometric morphometrics. *Annual Review of Anthropology* **36**(1), 261–281.

Spratt DM and Singleton GR (2001) Hepatic capillariasis. pp. 365–379. In Samuel WM, Pybus MJ and Kocan AA (Eds) *Parasitic diseases of wild mammal*. Iowa, Iowa State University Press.

Teta P, Abba AM, Cassini G, Flores DA, Galliari CA, Lucero S and Ramírez M (2018) Lista revisada de los mamíferos de Argentina [Revised list of the

mammals of Argentina]. *Mastozoología Neotropical* **25**(1), 163–198. [In Spanish.]

Udrizar Sauthier DE, Formoso AE, Andrade A, Podestá D and Teta P (2020) Key to cranial and mandibular remains of non-flying small mammals from southern South America. *Journal of Archaeological Science: Reports* **31**(1), 102310.

Valero MA, Darce NA, Panova M and Mas-Coma S (2001) Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Veterinary Parasitology* **102**(1–2), 85–100.

Valero MA, Panova M, Comes AM, Fons R and Mas-Coma S (2002) Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. *Journal of Parasitology* **88**(2), 308–313.

Valero MA, Perez-Crespo I, Periago MV, Khoubbane M and Mas-Coma S (2009) Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F. gigantica*. *Acta Tropica* **111**(2), 150–159.

Valero MA, Perez-Crespo I, Khoubbane M, Artigas P, Panova M, Ortiz P, Maco V, Espinoza JR and Mas-Coma S (2012) *Fasciola hepatica* phenotypic characterization in Andean human endemic areas: valley versus altiplanic patterns analysed in liver flukes from sheep from Cajamarca and Mantaro, Peru. *Infection, Genetics and Evolution* **12**(2), 403–410.

Valero MA, Bargues MD, Calderón L, Artigas P and Mas-Coma S (2018) First phenotypic and genotypic description of *Fasciola hepatica* infecting highland cattle in the state of Mexico, Mexico. *Infection, Genetics and Evolution* **64**(1), 231–240.