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# First morphogenetic analysis of parasite eggs from *Schistosomiasis haematobium* infected sub-Saharan migrants in Spain and proposal for a new standardised study methodology

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## Abstract

Schistosomiasis is a Neglected Tropical Disease caused by trematode species of the genus *Schistosoma*. Both, autochthonous and imported cases of urogenital schistosomiasis have been described in Europe. The present study focuses on eggs, considered pure *S. haematobium* by genetic characterisation (intergenic ITS region of the rDNA and *cox1* mtDNA). A phenotypic characterisation of *S. haematobium* eggs was made by morphometric comparison with experimental populations of *S. bovis* and *S. mansoni*, to help in the diagnosis of *S. haematobium* populations infecting sub-Saharan migrants in Spain. Analyses were made by Computer Image Analysis System (CIAS) applied on the basis of new standardised measurements and geometric morphometric tools. The principal component analysis (PCA), including seventeen non-redundant measurements, showed three phenotypic patterns in eggs of *S. haematobium*, *S. bovis* and *S. mansoni*. PCA showed that the *S. bovis* population presented a large egg size range with a pronouncedly larger maximum size. Similarly, *S. bovis* shows bigger spine values than *S. haematobium*. Mahalanobis distances between each pair of groups were calculated for each discriminant analysis performed. In general, *S. mansoni* and *S. bovis* present larger distances between them than with *S. haematobium*, i.e. they present the greatest differences. Regarding the spine, *S. haematobium* and *S. mansoni* are the most distant species. Results show the usefulness of this methodology for the phenotypic differentiation between eggs from these *Schistosoma* species, capable of discerning morphologically close eggs, as is the case of the *haematobium* group. *Schistosoma* egg phenotyping approaches may be applied to assess not only hybrid forms but also potential influences of a variety of other factors

## Keywords

*S. haematobium* *S. bovis*; *S. mansoni*; Eggs; Phenotypic analysis; Standardised methodology; Sub-Saharan migrants; Spain

## 1. Introduction

Schistosomiasis is a parasitic chronic disease caused by trematodes of the genus *Schistosoma*. This disease is considered a Neglected Tropical Disease (NTD) by WHO (WHO, 2020) in spite of having been ranked second only to malaria in socioeconomic terms and importance for public health. It affects more than 250 million people in 78 countries worldwide, 779 million are at risk of infection, and it is estimated to have caused 1.4 million disability-adjusted life years in 2017 (GBD 2017 DALYs and HALE Collaborators, 2018; Hotez et al., 2014; McManus et al., 2018).

There are six *Schistosoma* species known to affect humans: *Schistosoma haematobium*, *S. mansoni*, *S. intercalatum*, *S. guineensis*, *S. japonicum*, *S. mekongi*, some of them such as mainly *S. japonicum* but secondarily also *S. mansoni* being acknowledged to be zoonotic able to infect a broad range of livestock and wildlife animals (Colley and Secor, 2014; Webster et al., 2006; WHO, 2015).

*Schistosoma haematobium* has been reported in 54 countries, with more than 110 million people in Africa only (Boissier et al., 2016; McManus et al., 2018) and it is the most common species worldwide, i.e. more people are infected with *S. haematobium* than with all the other schistosome species combined (Boissier et al., 2016; Rollinson, 2009). Schistosomiasis is usually found in travellers and migrants in Europe (Lingscheid et al., 2017). Both autochthonous and imported cases of urogenital schistosomiasis have been described in Europe. In this sense, autochthonous cases were described in the French island of Corsica (Berry et al., 2014; Boissier et al., 2015), changing the paradigm of tropical diseases and rising awareness of the globalisation of pathogens. Moreover, molecular studies identified as aetiological agents of this introduction both pure *S. haematobium* and hybrids of *S. haematobium* and the ruminant parasite *S. bovis*. Furthermore, genetically pure *S. bovis* was also found in a patient of Corsica (Boissier et al., 2016).

On the other hand, *S. haematobium* cases in Spain have increased in the last decade (Salas-Coronas et al., 2020). Yet, studies that characterise genetically and phenotypically the aetiological agents in Europe are scarce.

The identification of schistosome species can be done indirectly, using molecular methods, but the direct identification of eggs in faeces or urine, based on their morphology, remains the cheapest, easiest and most viable way. Each trematode species has its own particular egg shape and the length and width of the eggs are generally within a specific range (Valero et al., 2009). These features allow for the aetiological diagnosis of schistosomiasis using easily and non-invasive obtained samples. In schistosomiasis, the usual diagnosis is based on the classification of eggs, found in, faeces and / or urine (WHO, 2020): *Schistosome* eggs have very characteristic shapes and features, with the morphology of the egg being diagnostic for each *Schistosoma* species (Candido et al., 2018), i.e. *S. mansoni* eggs show a prominent lateral spine, while those of *S. haematobium* present a terminal spine. On the other hand, *S. japonicum* eggs present only a rudimentary spine, varying from a small bump on the shell to a complete absence of this feature (Candido et al., 2018; Karl et al., 2013; Leiper, 1911; Woolley and Huffman, 1911). Likewise, different species of schistosomes present eggs that vary in size, not only between different species but within the same species of the *Schistosoma* parasite (Lewis and Tucker, 2014; Southgate and Bray, 2009). Until recent years, the tropism of egg excretion could be considered as a unique proxy for species identification. Eggs recovered from faeces were supposed to be *S. mansoni*, whereas eggs recovered from urine were supposed to be *S. haematobium*. However, since the discovery of several hybrid forms this proxy was challenged, and eggs with *S. mansoni* shape (*S. mansoni* x *S. haematobium* hybrids) can be found in urine and eggs with *S. haematobium* shape (*S. bovis* x *S. haematobium* hybrids) can be found in faeces (Huyse et al., 2009; Le Govic et al., 2019; Webster et al., 2019). The situation becomes much more complex if the parasite is a hybrid between animal and human infecting schistosomes, because egg shapes of animal schistosomes (e.g. *S. bovis* or *S. mattheei*) can be found in human excreta (Boissier et al., 2016; Huyse et al., 2009; Webster et al., 2019).

The *Schistosoma haematobium* group includes species that presents terminal spine eggs and develops in freshwater snails of the genus *Bulinus*. This group contains eight species, some of which are of medical (*S. haematobium*, *S. intercalatum*, *S. guineensis*) and veterinary (*S. bovis*, *S. mattheei* and *S. curassoni*) importance (Leger and Webster, 2017) and comprise a complex of species with a challenging diagnosis. The difficulty of egg species

differentiation is further complicated by the presence of intraspecific morphometric variation and potential natural hybridisations, as previously shown experimentally (Taylor, 1970; Wright and Southgate, 1976).

Morphological studies carried out to date with *S. haematobium*, *S. haematobium*-*S. bovis* hybrid eggs have failed to demonstrate morphological differences in shape and size between “pure” and hybrid eggs. Previous studies carried out both in adults and in eggs of flukes of other species between which hybridization takes place have shown that this possibility is feasible. These morphological studies in *F. hepatica* and *F. gigantica* in humans and animals show that the characterisation of pure standard populations facilitates subsequent comparisons and has made it even possible to identify intermediate morphological forms (Ashrafi et al., 2006; Periago et al., 2006, 2008; Valero et al., 2005, 2009; Afshan et al., 2014; Ahasan et al., 2016). Furthermore, studies in eggs of *Fasciola* species, show the influence of different factors on the morphological phenotype of parasite eggs, such as the geographical location or the host species (Valero et al., 2001, 2002, 2009). However, Fasciolidae eggs have different morphological characteristics than Schistosomatid eggs. Therefore, a search for a new analytical methodology that are useful is necessary for Schistosomatids.

Until now, there is no method available or measurement standardisation to properly analyse the phenotype of Schistosome eggs. Surprisingly, the particular morphology of *Schistosoma* eggs is mainly characterised by only three parameters, i.e. length, width, and the length/width ratio; the latter being highly variable (about 70% of this variation was present within a single patient) (Almeda et al., 1996). In addition, these classical morphometric measurements do not reveal the “spindle” character of some species. Therefore, with the aim of discerning *S. bovis*, *S. haematobium* and *S. mattheei* eggs, the width of the egg at an arbitrary distance of 50 µm from the non-spiked end was chosen (Alves, 1949). Later on, the width of the posterior process at 40 µm from the tip of the spine was introduced to characterise the eggs of the same species (Pitchford, 1965).

In the last years, geometric morphometrics has been proved as a useful tool for the differentiation of eggs of helminths such as the genus *Fasciola* (Valero et al., 2009) or *Trichuris* (García-Sánchez et al., 2020). As previously suggested, the egg shape of *Schistosoma* spp. could be determined more precisely by taking additional measurements and by using geometric morphometric techniques (Boon et al., 2017).

The objectives of this work are: i) to characterise morphogenetically eggs of *S. haematobium* involved in cases of sub-Saharan migrants present in Spain by means of a morphometric comparison with pure experimental populations of *S. bovis* and *S. mansoni*; and ii) to propose a new analytical methodology. In order to do so, only eggs considered pure *S. haematobium* by genetic characterisation (ITSs of rDNA and *cox1* mtDNA) have been used.

## 2. Material and methods

### 2.1. Ethics statement

Human samples were obtained at the Tropical Medicine Unit of Poniente Hospital (El Ejido, Almería, Spain) for diagnostic purposes as part of the standard protocol care for sub-Saharan patients attended at such a unit. Anonymised data were collected retrospectively. This study was approved by the Ethics Committee of the Coordinating Site (protocol Schis-01-UMT-2018). At the end of the study, all participants were informed about their parasitological results and free treatment was offered when found positive for schistosomiasis (single 40 mg/kg dose of Praziquantel).

Animal samples were obtained at the laboratory of the IHPE (Hôtes-Pathogènes-Environnements), CNRS, Université de Perpignan (Perpignan, France) which has permission A66040 from «Ministère de l’Enseignement supérieur de la Recherche et de l’Innovation (France)» for animal experimentation. Experimenters hold the certificate for animal experimentation (authorisation 007083, decree 87-848 and 2012201–0008). Housing, breeding and animal care followed the national and European ethical requirements.

## 2.2. Material

A total of 174 *Schistosoma* eggs were analysed (Table 1):

*S. haematobium* obtained from urine (humans): 84 eggs from six urine samples of young migrant men (20–27 years old, average 24; 5 from Senegal, 1 from Mauritania) living in Spain (from 1 to 14 months) and diagnosed with urogenital schistosomiasis at the Tropical Medicine Unit of Poniente Hospital (El Ejido, Almeria, Spain) and considered pure *S. haematobium* by genetic characterisation (intergenic ITS region of rDNA and *cox1* mtDNA). Epidemiological and clinical records were also collected for each patient. Parasite eggs were recovered from urine by using a 40 µm cell strainer (Falcon®) and 0.9% NaCl saline solution to wash the sample through the filter. All eggs trapped on the strainer were collected in a Petri dish containing 20 mL of 0.9% saline solution.

*S. bovis* obtained from livers (hamster): 47 eggs from infected hamster liver squashes from the laboratory strain (Silva et al., 1977), identified as genetically pure, maintained in the IHPE (Interactions Hôtes-Pathogènes-Environnements), CNRS, Perpignan, France. They were mashed and passed through a column of metal sieves of different pore size to isolate parasite eggs from the debris. All eggs trapped on the last sieve were collected in a Petri dish containing 20 mL of 0.9% saline solution.

*S. mansoni* obtained from livers (mice): 43 eggs from infected mice liver squashes from the laboratory strain (SmbRE strain, Brazil) (Theron et al., 1997), identified as genetically pure, maintained in the IHPE (Interactions Hôtes-Pathogènes-Environnements), CNRS, Perpignan, France. Parasite eggs were recovered from the livers following the same technique and procedure as described above for *S. bovis*.

This study has its limitations. It must be taken into consideration that there might be differences in the size and shape of eggs depending on their method of sampling as geographical origin. As previously highlighted (Touassem, 1987), the shape of *S. bovis* eggs from the host faeces (the typical ones) and from the distal portion of the worm uterus tended to be homogeneous, whereas those from the host liver were more heterogeneous for two different strains of this species. Previous studies of *Fasciola hepatica* egg size showed characteristic morphometric traits to be related to their definitive host species (Valero et al., 2009). Nevertheless, this phenomenon has not been previously described in the genus *Schistosoma*. *Schistosoma bovis* and *S. mansoni* eggs used in this work were recovered from hamster livers and not from natural host samples, which might have influenced the morphometry outcome. Nevertheless, the high specificity of pure *S. bovis* for ruminant host makes it difficult to plan a study design with samples of human origin (as only sporadic cases of *S. bovis* have been detected in humans, with a very small burden). For this reason and given the need to use genetically characterised material, experimental *S. bovis* eggs from hamsters were used. On the other hand, pure *S. mansoni* eggs, were used in this study as external group, as these eggs have a very different morphology (lateral spine). The egg sample was obtained from mice, a highly validated experimental model.

Furthermore, egg sizes obtained herein experimentally have been verified to agree with previous egg descriptions from humans (see discussion). However, we consider size and shape of these three species to be truly different, so that the influence of host and sample methods can be disregarded.

As fixation is known to affect size and shape of helminth eggs, egg materials used in the present study were analysed without prior fixation, suspended in 0.9% NaCl saline solution, preserved in darkness at 4 °C until required and digitalised in the shortest possible time, within the next fortnight after collection (Valero et al., 2009).

## 2.3. Molecular analysis of *Schistosoma* eggs from urine samples

*S. haematobium*-like eggs obtained from the six human urine samples were molecularly characterised by a mitochondrial marker (partial cytochrome c oxidase subunit I - *cox1*), and a nuclear marker (intergenic ITS region of the rDNA). Genomic DNA was extracted individually from each egg using the InstaGene™ Matrix kit (Bio-Rad Laboratories® CA, USA) following the manufacturer's instructions, and stored at – 20 °C until use.

For mitochondrial *cox1* profiling, a RD-PCR from each of the 84 eggs was performed using species-specific primers (Boon et al., 2017; Van den Broeck et al., 2011; Webster et al., 2010) to amplify a specific *cox1* DNA region (differing in length) for *S. bovis* (306 bp), *S. mansoni* (375 bp) and *S. haematobium* (543 bp). The PCR reactions were performed in a final volume of 12.6  $\mu$ L, comprising 8  $\mu$ L of DNA template, 2.5  $\mu$ L of 5X colorless GoTaq Flexi Buffer (Promega), 0.75  $\mu$ L of MgCl<sub>2</sub> (25 mM), 0.25  $\mu$ L of dNTP (10 mM), 1  $\mu$ L of primer mix (1  $\mu$ M), and 0.1  $\mu$ L of Go Taq G2 Hot Start Polymerase (Promega). The PCR conditions were 3 min at 95 °C, followed by 35cycles of 30 s at 94 °C, 1 min 30 s at 58 °C and 1 min 30 s at 72 °C followed by a final cycle at 72 °C for 7 min. The *cox1*-PCR products were visualised for electrophoresis on a 2.5% agarose gel stained with GelRed (Biotium) and photographs were taken using the UVP xgel documentation system.

To complement the information provided by mitochondrial DNA profiling, inherited maternally, the complete ITS-1–5.8S-ITS-2 of the rDNA were PCR amplified independently and each PCR product was sequenced from a subsample of 25 eggs (4–6 eggs/ per patient), using BD1 and BD2 primers (Luton et al., 1992). PCR amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA), following PCR conditions previously described (Bargues et al., 2017; Chougar et al., 2020). PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol and resuspended in 50  $\mu$ l of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm on an Eppendorf BioPhotometer (Hamburg, Germany). Sequencing was performed on both strands by the dideoxy chain-termination method. It was carried out with the Taq dye-terminator chemistry kit on an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) using PCR primers.

Sequences were edited and assembled using Sequencher v.5.4.6 (Gene Codes Co.) and aligned with MEGA version 7 (Kumar et al., 2016), using default settings. A careful inspection of all nucleotide positions in the raw sequence chromatograms allowing the detection of sequence polymorphisms between *S. haematobium* and *S. bovis*, was checked to identify possible heterogeneity, as previously described (Angora et al., 2020; Savassi et al., 2020; Webster et al., 2013). Homologies were performed using the BLASTN program from the National centre for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>).

#### 2.4. Schistosome egg digitalization and measurements

Schistosome egg measurements were performed on 174 samples [84 eggs from human urine (*Sh*), 47 from a hamster liver (*Sb*), and 43 from a mouse liver (*Sm*)] (Table 1). The measurements were taken by means of a Computer Image Analysis System (CIAS), including a computer connected to a digital camera linked to a microscope. Each egg was individualised in a 0.9% NaCl drop on a slide and photographed using a microscope (Leica DMR 72-89663) with 400 magnifications and the high-resolution camera Leica DFC450C controlled by the LAS software. Representative images of the photographed eggs and their spines are shown in Fig. 1.

All pictures were taken without coverslip for the establishment of a correct standardisation of the measurements. During the workup of the standardisation, the influence of the coverslip on egg shape and size was assessed. The same egg was photographed before (Fig. 1D) and after (Fig. 1E) a cover slip was gently lowered on the saline solution drop where it was placed. No pressure was applied. In addition, the lack of coverslip allowed the recovery of individual eggs, and preservation in EtOH 70°, for posterior molecular characterisation. For morphometric characterisation, the software Image Pro-Plus v5.1 (Media Cybernetics, USA) was used.

A total of twenty-five measurements were designed to phenotypically characterise the different schistosome eggs. Twenty-five measurements were designed. In order to identify the ones that give some valuable information about the egg morphology and therefore, were suitable for the principal component analysis, an iterative process of measurement combinations was performed. As a result, 17 measurements were considered of interest as they yield

high contribution values in the multivariate analysis. These 17 selected measurements are detailed in Fig. 2 and include:

- **Egg Area (EA)**. Area included in the polygon defining the egg outline.
- **Egg Perimeter (EP)**. Length of the egg outline.
- **Radius max (Rmax)**. Maximum distance between egg centroid and outline.
- **Radius min (Rmin)**. Minimum distance between egg centroid and outline.
- **Egg length (EL)**. Feret diameter along major axis of egg.
- **Egg width (EW)**. Feret diameter along minor axis of egg.
- **Egg Roundness (ER)**. Defined as  $(\text{perimeter}^2/4\pi \cdot \text{Area})$ . 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 (Anonymous, 2001; Ohnuma et al., 2006).
- **Width at 20  $\mu\text{m}$  from blunt end (BE20)**.
- **Width at 35  $\mu\text{m}$  from blunt end (BE35)**.
- **Width at 50  $\mu\text{m}$  from blunt end (BE50)**.
- **Width at 20  $\mu\text{m}$  from spine end (SE20)**.
- **Width at 35  $\mu\text{m}$  from spine end (SE35)**.
- **Width at 40  $\mu\text{m}$  from spine end (SE40)**.
- **Spine length (SL)**.
- **Spine base width (SBW)**.
- **Spine medium width (SMW)**. - **Spine end width (SEW)**.

The above mentioned 17 measurements included size, shape and spine features and were not redundant (EA, EP, Rmax, Rmin, EL, EW, ER, BE20, BE35, BE50, SE20, SE35, SE40, SL, SBW, SMW and SEW). Furthermore, the following ratios were used:

**Egg Shape Ratio (ESR)**. Defined as  $(\text{EL}/\text{BE35} \cdot \text{Roundness})$ . It is a measurement of how spindled an object is, as it relates the width of the egg at 35  $\mu\text{m}$  from the blunt end (where the narrowing is usually found) with its total length and circularity.

- **Elongation Ratio (ERatio)**. Defined as  $(\text{Rmax}/\text{Rmin})$ .

## 2.5. Statistical data and analyses

Statistical analysis was performed using SPSS Statistics 26. Comparison of the average egg measurements from the different populations was carried out using one-way ANOVA and the Bonferroni test. Values were considered statistically significant when  $P < 0.05$ .

The software Ene 3.0 (Servei d'Estadística Aplicada, Universitat Autònoma de Barcelona, Cerdanyola del Valles, Spain, distributed by GlaxoSmithKline) was used to establish the minimum number of eggs needed to obtain statistically significant results ( $P: 0.05$ , power: 80%).

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 a common allometric trend is identifiable (Rohlf and Marcus, 1993). Biometric data was examined using multivariate analysis. Morphological differences were quantified by geometrical morphometrics, which includes validated methods for analysing variation in organismal shape (Klingenberg, 2016). 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).

Principal component analysis (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 each analysis, we built a two-dimensional morphospace plot based on the first two principal component.

Size-free canonical discriminant analysis was used on the covariance matrix of measurements transformed into logarithms to assess morphometric variation amongst 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).

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 can be used to measure how distant a point is from the centre of a multivariate normal distribution. The degree of similarity between egg populations was assessed through pairwise Mahalanobis distances (García-Sánchez et al., 2020).

PCA was carried out using the natural logarithm of measurements applying the BAC software. PVA and Mahalanobis distances were calculated using PAD software and tested by non-parametric permutation tests with 1000 iterations each. Values were considered statistically significant when  $P < 0.05$  (Dujardin, 2002; García-Sánchez et al., 2019). Nevertheless, to avoid matrix singularities, some measures were discarded because of their partial overlap with other measures. The following 17 non-redundant measurement (i.e. one is not included in another one) were considered: EA, EP, Rmax, Rmin, EL, EW, BE20, BE35, BE50, SE20, SE35, SE40, ER, SL, SBW, SMW and SEW.

Linear regression was calculated for the ERatio values and PC1 and PC2 (SPSS Statistics 26). This morphometric trait has been used in egg characterisation of other animal species, proving to be a useful tool of classification (Attard et al., 2018). Results were considered statistically significant when  $P < 0.05$ .

### 3. Results

#### 3.1. Molecular criteria for species identification

Genetic profiles provided by *cox1* mtDNA and ITS region of rDNA, allowed defining that the parasites (schistosome terminal-spined eggs from urine samples) were *S. haematobium* and not a hybrid species.

##### 3.1.1. *Cox1* rapid diagnostic PCR

The 84 eggs from the urine samples from the six *Schistosoma*-infected patients furnished an *S. haematobium cox1* profile. No egg/miracidium gave an *S. bovis* or *S. mansoni* profile.

##### 3.1.2. ITS rDNA sequence analysis

ITS rDNA profiles showed that *S. haematobium* were present in all the six human samples selected for this study. No hybrid profiles were detected in the chromatograms. amongst the 25 sequences obtained for the nuclear Internal Transcribed Spacers (ITSs), two profiles of ITSs were retrieved; one of them (Sh-1A) showed sequence identity (100%) with *S. haematobium* (consensus sequence retrieved from GenBank = Acc. Nos. GU257398, JQ397400-JQ397414, FJ588861, MT884914 and MT158873) and was obtained in 17 of 25 sequences (68%), and in patients from both, Mauritania and Senegal. The second profile (Sh-2A) presented a heterozygotic signal (C/T) in position 533 of the ITSs alignment and did not correspond to any discriminative position amongst other *Schistosoma* species with terminal-

spined eggs. This Sh- 2A sequence was present in 32% (8/25) of samples, and only in two patients from Senegal, and showed sequence identity (100%) with *S. haematobium* from the Ivory Coast (MG554667).

### 3.2. Comparative biometric analysis amongst *Schistosoma* species

Biometric features were compared between different species (Table 2). As previously known, the longest eggs belong to *S. bovis*, while the widest are those of *S. mansoni*. The overlap in the length range of some *S. haematobium* and *S. bovis* eggs is noteworthy, being inexistent between the latter and *S. mansoni* eggs.

The significant differences obtained by comparing each egg measurement in pairs of *Schistosoma* species using the Bonferroni tests are shown in Table 3. *S. haematobium* eggs are significantly different from those of *S. bovis* and *S. mansoni* for Egg Area (EA), Egg Perimeter (EP), Width at 50  $\mu\text{m}$  from Blunt End (BE50), Width at 20  $\mu\text{m}$  from Spine End (SE20), Width at 35  $\mu\text{m}$  from Spine End (SE35), Width at 40  $\mu\text{m}$  from Spine End (SE40), Egg Shape Ratio (ESR), Spine Length (SL), Spine Base Width (SBW), Spine Medium Width (SMW), and Spine End Width (SEW).

Eggs of *S. bovis* are significantly different from those of *S. mansoni* regarding Radius maximum (Rmax), Egg Perimeter (EP), Egg Length (EL), Egg Width (EW), Egg Roundness (ER), Width at 20  $\mu\text{m}$  from Blunt End (BE20), Width at 35  $\mu\text{m}$  from Blunt End (BE35), Width at 50  $\mu\text{m}$  from Blunt End (BE50), Width at 20  $\mu\text{m}$  from Spine End (SE20), Width at 35  $\mu\text{m}$  from Spine End (SE35), Width at 40  $\mu\text{m}$  from Spine End (SE40), Egg Shape Ratio (ESR), Spine Length (SL), Spine Base Width (SBW), Spine Medium Width (SMW) and Spine End Width (SEW). These results suggest that the morphological traits designed offer detailed and specific values for each schistosome species proving to be useful to characterise and compare them.

### 3.3. Multivariate analysis amongst species

First, a principal component analysis was performed. Initially, the 17 non-redundant measurements were analysed all together (EA, EP, Rmax, Rmin, EL, EW, ER, BE20, BE35, BE50, SE20, SE35, SE40, SL, SBW, SMW and SEW), regardless of the nature of each of them (size, shape, spine) (Fig. 3A). The three species appear well differentiated in the factor map, i.e. three phenotypic patterns could be distinguished in the egg material analysed: *S. haematobium*, *S. bovis* and *S. mansoni*. PCA showed that the *S. bovis* population presented a large egg size range with a pronouncedly larger maximum size. Contribution of the two principal components was PCI: 57% and PCII: 30%. Moreover, for subsequent analysis, measurements were divided into three groups: size, shape and spine measurements. Size measurements included EA, Rmax, Rmin, EP, EL and EW. *S. haematobium* and *S. mansoni* mostly overlapped as their eggs have a similar size, slightly bigger for *S. mansoni* (Fig. 3B). Shape measurements included the Width at six different distances from both the Spike and Blunt End of the eggs (BE20, BE35, BE50, SE20, SE35 and SE40) and ER (Fig. 3C). In this factor map, *S. haematobium* is closer to *S. bovis* although a certain overlap occurs with *S. mansoni*. Spine measurements included SL, SBW, SMW and SEW (Fig. 3D) and show clear differences between *S. mansoni* and the other two species. Despite the overlap between *S. haematobium* and *S. bovis*, the latter show larger spine values than *S. haematobium* when projected on the X axis.

Second, a multivariate discriminant analysis of the seventeen non-redundant measurements was performed (EA, EP, Rmax, Rmin, EL, EW, ER, BE20, BE35, BE50, SE20, SE35, SE40, SL, SBW, SMW and SEW) (Fig. 4A). In this case, canonical factor contribution was significant, and the three species appear well differentiated. Therefore, no more analyses were needed. Spine measurements were also included for a detailed description (Fig. 4B). The contribution of canonical factors increased for this analysis and species appeared differently, appearing closer together (even overlapping) but still well differentiated.

Mahalanobis distances between each pair of groups were calculated for each discriminant analysis performed (Table 4). In general (Table 4A = 17 measurements: (EA, EP, Rmax, Rmin, EL, EW, ER, BE20, BE35, BE50, SE20, SE35, SE40, SL, SBW, SMW and SEW), *S. mansoni* and *S. bovis* present larger distances between them than with *S.*

*haematobium*, i.e. they present the greatest differences. Regarding the spine, *S. haematobium* and *S. mansoni* are the most distant species (Table 4B = spine measurements: SL, SBW, SMW and SEW).

### 3.4. Linear regression analysis

The relationship between the elongation ratio (ERatio) values and aforementioned PC1 and PC2 scores was represented through a linear regression analysis, one for each combination of measurements.

- **Size, shape and spine measurements** (17 measurements: EA, EP, Rmax, Rmin, EL, EW, ER, BE20, BE35, BE50, SE20, SE35, SE40, SL, SBW, SMW and SEW). PC1 scores (57%) showed a significant positive correlation with ER values for the eggs of *S. haematobium* ( $rs=0.0855$ ,  $P = 0.0070$ ), *S. bovis* ( $rs=0.3374$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.1519$ ,  $P = 0.0098$ ) (Fig. 5A). PC2 scores (30%) were significantly negatively correlated with ER values for eggs of *S. haematobium* ( $rs=0.6249$ ,  $P<0.0001$ ), *S. bovis* ( $rs=0.2331$ ,  $P=0.0006$ ) and *S. mansoni* ( $rs=0.5464$ ,  $P<0.0001$ ) (Fig. 5B).

- **Size and shape measurements** (13 measurements: EA, EP, Rmax, Rmin, EL, EW, BE20, BE35, BE50, SE20, SE35, SE40 and ER). PC1 scores (77%) showed a significant positive correlation with ER values for eggs of *S. haematobium* ( $rs=0.7787$ ,  $P<0.0001$ ), *S. bovis* ( $rs=0.6754$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.6981$ ,  $P<0.0001$ ) (Fig. 6A). PC2 scores (14%) were equally significantly positively correlated with ER values for eggs of *S. bovis* ( $rs=0.3494$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.1507$ ,  $P = 0.0101$ ), whereas no correlation was detected for *S. haematobium* eggs ( $rs=0.000667$ ,  $P = 0.8156$ ) (Fig. 6B).

- **Size measurements** (6 measurements: EA, EP, Rmax, Rmin, EL and EW). PC1 scores (70%) showed a significant and strong negative correlation with ER values for eggs of *S. bovis* ( $rs=0.4154$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.184$ ,  $P = 0.0035$ ), whereas no correlation was found for *S. haematobium* eggs ( $rs = 0.004829$ ,  $P = 0.5299$ ) (Fig. 7A). On the contrary, PC2 scores (27%) were significantly positively correlated with ER values of all species: *S. haematobium* ( $rs=0.8695$ ,  $P<0.0001$ ), *S. bovis* ( $rs=0.8799$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.8295$ ,  $P<0.0001$ ) (Fig. 7B).

- **Shape measurements** (7 measurements: ER, BE20, BE35, BE50, SE20, SE35 and SE40). PC1 scores (88%) showed a significant and strong positive correlation with ER values for eggs of all species: *S. haematobium* ( $rs=0.7256$ ,  $P<0.0001$ ), *S. bovis* ( $rs = 0.6358$ ,  $P<0.0001$ ) and *S. mansoni* ( $rs=0.6425$ ,  $P<0.0001$ ) (Fig. 8A). A significant negative correlation was found for PC2 scores (10%) with ER values for *S. haematobium* eggs ( $rs=0.08718$ ,  $P = 0.0064$ ), whereas no correlation was found neither for eggs of *S. bovis* ( $rs=0.002111$ ,  $P = 0.7591$ ) nor *S. mansoni* ( $rs=0.07454$ ,  $P = 0.0765$ ) (Fig. 8B).

The results obtained in the lineal analysis show different correlation patterns amongst the *Schistosoma* species. Although *S. bovis* and *S. mansoni* are the most distant species in terms of taxonomy and morphology, they present a similar behaviour in terms of correlation of their principal components and elongation ratios when size and shape measurements are analysed. However, *S. haematobium* behaves differently than the other two species, showing no correlation when size measurements are involved, while being significantly correlated when only shape measurements are considered.

## 4. Discussion

### 4.1. Morphogenetic characteristics of *S. haematobium*

This is the first morphogenotypic study to characterise *S. haematobium* eggs thoroughly analysis. Genetic profiles provided by the *cox1* mtDNA and the intergenic ITS region of the rDNA allowed defining that the parasites (schistosome terminal-spined eggs from urine samples) were *S. haematobium* and not a hybrid species.

Morphological traits of *Schistosoma* eggs have been characterised by using, for the first time, 19 parameters, which allow increasing the specific accuracy substantially when compared to classical measurements, i.e. length and width.

Furthermore, this technique has been applied in *S. bovis* and *S. mansoni*. The latter also plays the role of external group.

*S. haematobium* and *S. bovis* eggs are both terminal-spined but significant differences are noteworthy. *S. bovis* eggs released in stool are larger (usually between 200 - 300  $\mu\text{m}$ ) and spindle-shaped, one bearing a well differentiated spine, the other evenly rounded (Boon et al., 2017; Taylor, 1970; Touassem, 1987). Previous descriptions of *S. bovis* egg showed a wide range in the measurements included (Table 5). Despite the fact that the eggs analysed herein come from hamsters, their length range (162.63 - 251.16  $\mu\text{m}$ ) fits perfectly with depictions of cattle samples 130 – 300  $\mu\text{m}$  (MacHattie et al., 1933; Savassi et al., 2020), suggesting that differences found between parasite species eggs are minimally influenced by the host species. Due to the spindle shape of *S. bovis*, their maximum width of 40–90  $\mu\text{m}$  (Alves, 1949; Savassi et al., 2020) and 35.65 - 81.21  $\mu\text{m}$  (present study) does not differ significantly from that of *S. haematobium* of 38–95  $\mu\text{m}$  (Savassi et al., 2020; Zeibig, 2012) and 38.17 - 66.85  $\mu\text{m}$  (present study), providing further evidence that this measurement on its own is insufficient for a detailed characterisation. The length/width ratio of the eggs analysed herein (data not shown) fits within the ratios previously described (Table 5).

The preceding width measurements proposed at 40  $\mu\text{m}$  and 50  $\mu\text{m}$  (Alves, 1949; Pitchford, 1965) were designed especially for *S. mattheei* phenotyping. 40  $\mu\text{m}$  was longer than the length of the longest spine of *S. mattheei* measured at their laboratory and fell well within that portion of the egg where the shouldering would affect the shape. These two measurements seem sufficient for the characterisation of *S. bovis* and *S. mattheei* although width at a greater distance from blunt and spike ends of *S. bovis* eggs should be considered as differences that can usually be found closer to the halfway point. However, when applying them to *S. haematobium* eggs, relevant information about their shape would be missing, as 50 and 40  $\mu\text{m}$  are too close to the egg midway and, therefore, yield a very similar value to their maximum width. In this study, four shorter measurements have been included, allowing a thorough description of the egg shape. However, when comparing exclusively *S. bovis* populations, width at 70 and 60  $\mu\text{m}$  from blunt and spine end, is highly recommended in order to detect fluctuations that might be present in different strains.

*S. haematobium* eggs are smaller and rounder than *S. bovis* and are usually found in urine. In previous descriptions, length varied from 83  $\mu\text{m}$  to 170  $\mu\text{m}$  in eggs originating from human urine (Table 6) (Alves, 1949; Pitchford, 1965; Savassi et al., 2020). The average size (139.13  $\mu\text{m}$ , range 111.64 - 164.12  $\mu\text{m}$ ) of the *S. haematobium* eggs analysed herein falls within this range although our minimum record is slightly larger. The maximum width (66.85  $\mu\text{m}$ ) detected in the *S. haematobium* eggs analysed in the present study is also significantly smaller than previously described (95  $\mu\text{m}$ ) (Savassi et al., 2020), probably due to the use of the coverslip in similar studies.

Previous descriptions showed a wide range in the measurements included, indicating the necessity of further studies on the influence of the different factors involved such as geographical origin.

Earlier phenotypic characterisations of *S. mansoni* eggs by length, width and spine length show similar values to those yielded in the present study (Table 7). Length ranges vary from 112 up to 180  $\mu\text{m}$  (present study: 106.55 - 156.53  $\mu\text{m}$ ) and width from 40 to 73  $\mu\text{m}$  (present study: 43.41 - 77.51  $\mu\text{m}$ ) (Candido et al., 2018; CDC, 2019; de Souza et al., 2019; Lenzi et al., 2008). The length/width ratio obtained in *S. haematobium* eggs analysed in this study agrees with other reports (CDC, 2019), but it is slightly smaller than the rest of descriptions (i.e. a smaller difference between length and width are present). Regarding the spine, *S. haematobium* eggs analysed in this study show a larger average spine (25.06  $\mu\text{m}$ ) when compared to previous descriptions but similar to a hybrid strain reported in the Ivory Coast (Depaquit et al., 2019). The statistically significant variability found in three different Brazilian strains is noteworthy, evidencing intraspecific variation, including the influence of the geographical origin on *Schistosoma* species (Euzebio et al., 2012).

Egg variability associated to geographical origin has previously been described in other trematodes such as *F. hepatica* and *F. gigantica* (Valero et al., 2005, 2009). In the genus *Schistosoma*, intraspecific egg variability has been described in few species. The variability in size and shape of *S. intercalatum* eggs from Sao Tom were represented an obstacle in its differentiation from *S. haematobium* eggs through microscopical observations in that region. The results showed that this variation is not related to age, sex or intensity of infection (Almeda et al., 1996). Egg variability in size has also been found within two populations of *S. haematobium* from Algeria (Kechemir and Theron, 1997), but studies on this species remain scarce.

Consequently, the variability in size and shape of *Schistosoma* eggs highlights the necessity of having standardised measurements available which enable detailed morphological analysis.

#### 4.2. Proposal of a new analytical methodology

Most of the scientific work published in the last decade about *Schistosoma* species is based on molecular characterisation. Herein, we propose a wide selection of morphometric traits that may complement molecular tools. Schistosome eggs have specific morphological features which play a central role in the diagnosis of the disease. Although several studies have been carried out to elucidate morphological characteristics of the eggs, none of them has proposed a standardised methodology capable of discerning morphologically close eggs as is the case inside the *haematobium* group.

Both *S. haematobium* and *S. bovis* belong to the *haematobium* group complex. Despite the differences found in average size and shape of eggs and spines at first sight, the length and width ranges of these species overlap. Furthermore, crossing experiments between *S. haematobium* and *S. bovis* resulted in eggs with intermediate sizes and shapes (Bremond et al., 1993 ; Taylor, 1970).

The present study proposes a new standardised approach able to discern clearly amongst pure species. As a matter of fact, without having previously demonstrated this possibility, the differentiation of similar eggs such as hybrid forms cannot be addressed. In this sense, the results obtained in the present work are expected to lay the groundwork of comparative morphometric studies, making the differentiation of even potential intermediate forms possible.

Additionally, these new morphometric concepts provide appropriate tools to characterise *Schistosoma* egg morphological features in order to evaluate the influence of the different factors on the morphological phenotype of parasite eggs such as the geographical location.

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### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Table 1**

Number of eggs phenotyped of each species and their origin.

Species	Country	Sample/Host /Patient code	No. eggs phenotyped
<b><i>S. haematobium</i></b> <b>(Sh)</b>	<b>MAURITANIA</b>	Urine/Human/P-	<b>14</b>
	<b>SENEGAL</b>	15	12
		Urine/Human/P-4	
		Urine/Human/P-	30
		16	
		Urine/Human/P-	6
		18	
Urine/Human/P-	7		
	19		
	Urine/Human/P-	15	
	21		
	<i>Total</i>		<b>84</b>
<b><i>S. bovis</i></b> ( <b><i>Sb</i></b> )	<b>SPAIN</b>	Liver/Hamster/Lab strain <i>Sb</i>	<b>47</b>
<b><i>S. mansoni</i></b> <b>(Sm)</b>	<b>BRAZIL</b>	Liver/Mouse/Lab. Strain <i>Sm</i>	<b>43</b>

**Table 2**Biometric data of the three schistosome species eggs. Mean  $\pm$  SD (Min – Max).

	<i>S. haematobium</i>	<i>S. bovis</i>	<i>S. mansoni</i>
Egg Area (EA)	5027.1 $\pm$ 791.1 (2890.27 - 6494.93)	5901.72 $\pm$ 1807.79 (2962.75 - 11,071.52)	6104.77 $\pm$ 1042.86 (3314.58 - 7562.29)
Radius maximum (Rmax, $\mu$ m)	72.52 $\pm$ 6.65 (56.94 - 85.57)	103.22 $\pm$ 11.69 (82.88 - 131.61)	73.59 $\pm$ 6.41 (57.66 - 83.76)
Radius min (Rmin, $\mu$ m)	25.89 $\pm$ 2.99 (17.23 - 33.12)	26.6 $\pm$ 5.21 (16.87 - 40.14)	27.82 $\pm$ 3.33 (18.41 - 33.37)
Egg Perimeter (EP, $\mu$ m)	309.4 $\pm$ 27.22 (252.45 - 355.84)	425.95 $\pm$ 48.95 (344.49 - 539.46)	349.1 $\pm$ 27.46 (266.79 - 392.71)
Egg Length (EL, $\mu$ m)	139.13 $\pm$ 13.48 (111.64 - 164.12)	201.41 $\pm$ 22.61 (162.63 - 251.16)	136.69 $\pm$ 11.94 (106.55 - 156.53)
Egg Width (EW, $\mu$ m)	53.95 $\pm$ 5.58 (38.17 - 66.85)	55.44 $\pm$ 10.4 (35.65 - 81.21)	64.73 $\pm$ 6.57 (43.41 - 77.51)
Egg Roundness (ER)	1.54 $\pm$ 0.14 (1.25 - 1.82)	2.55 $\pm$ 0.3 (2 - 3.42)	1.62 $\pm$ 0.11 (1.41 - 1.88)
Width at 20 $\mu$ m from Blunt End (BE20, $\mu$ m)	31.18 $\pm$ 5.65 (20.2 - 42.53)	15.54 $\pm$ 1.79 (11.96 - 18.87)	30.73 $\pm$ 3.73 (24.18 - 39.88)
Width at 35 $\mu$ m from Blunt End (BE35, $\mu$ m)	39.35 $\pm$ 7.26 (24.45 - 53.41)	18.73 $\pm$ 2.08 (14.62 - 23.12)	41.87 $\pm$ 4.46 (31.63 - 51.28)
Width at 50 $\mu$ m from Blunt End (BE50, $\mu$ m)	46.83 $\pm$ 7.5 (31.09 - 62.19)	23.31 $\pm$ 3.41 (17.01 - 29.5)	50.53 $\pm$ 4.69 (39.06 - 59.25)
Width at 20 $\mu$ m from Spine End (SE20, $\mu$ m)	24.8 $\pm$ 4.62 (16.74 - 38.25)	14.24 $\pm$ 2.9 (7.71 - 20.73)	51.09 $\pm$ 9.56 (32.41 - 65.09)
Width at 35 $\mu$ m from Spine End (SE35, $\mu$ m)	39.18 $\pm$ 4.81 (30.56 - 54.75)	22.23 $\pm$ 3.81 (14.62 - 31.63)	54.19 $\pm$ 5.43 (36.66 - 61.66)
Width at 40 $\mu$ m from Spine End (SE40, $\mu$ m)	43.25 $\pm$ 4.81 (34 - 57.94)	23.92 $\pm$ 4.31 (14.35 - 34)	54.76 $\pm$ 5.54 (38.25 - 63.25)
Length/Width Ratio (L/W)	2.6 $\pm$ 0.3 (1.99 - 3.31)	3.69 $\pm$ 0.42 (3.03 - 4.73)	2.12 $\pm$ 0.18 (1.76 - 2.56)
Egg Shape Ratio (ESR)	2.36 $\pm$ 0.38 (1.7 - 3.16)	4.28 $\pm$ 0.59 (3.19 - 5.77)	2.03 $\pm$ 0.18 (1.71 - 2.49)
Elongation Ratio (ERatio, Rmax/Rmin)	2.83 $\pm$ 0.36 (2.12 - 3.72)	3.96 $\pm$ 0.50 (3.28 - 5.36)	2.67 $\pm$ 0.24 (2.19 - 3.25)
Spine Length (SL, $\mu$ m)	6.55 $\pm$ 2.09 (3.19 - 11.32)	12.55 $\pm$ 2.95 (5.71 - 18.07)	25.06 $\pm$ 3.66 (17.3 - 31.56)
Spine Base Width (SBW, $\mu$ m)	4.68 $\pm$ 1.1 (2.13 - 7.56)	5.89 $\pm$ 1.8 (2.66 - 9.03)	20.06 $\pm$ 3.36 (13.93 - 29.25)
Spine Medium Width (SMW, $\mu$ m)	2.8 $\pm$ 0.69 (1.19 - 4.45)	3.46 $\pm$ 1.18 (1.59 - 6.11)	10.07 $\pm$ 1.69 (6.03 - 13.22)
Spine End Width (SEW, $\mu$ m)	1.54 $\pm$ 0.37 (0.59 - 2.67)	1.35 $\pm$ 0.41 (0.59 - 2.94)	2.2 $\pm$ 0.5 (1.13 - 3.1)

**Table 3**

Significantly different measurements amongst the three schistosomes species eggs by Bonferroni tests ( $P < 0.05$ ).

	<i>S. bovis</i>	<i>S. mansoni</i>
<b><i>S. haematobium</i></b>	EA, Rmax, EP, EL, L/W, ER, ERatio, BE20, BE35, BE50, SE20, SE35, SE40, ESR, SL, SBW, SMW, SEW.	EA, Rmin, EP, EW, L/W, BE50, SE20, SE35, SE40, ESR, SL, SBW, SMW, SEW.
<b><i>S. bovis</i></b>		Rmax, EP, EL, EW, L/W, ER, ERatio, BE20, BE35, BE50, SE20, SE35, SE40, ESR, SL, SBW, SMW, SEW.

**Table 4**

Mahalanobis distances between the three *Schistosoma* species (*S. haematobium*, *S. bovis* and *S. mansoni*) for the two discriminant analysis performed: (A) all measurements (17) and (B) spine measurements (4). All distances were statistically significant.

A	<i>S. haematobium</i>	<i>S. bovis</i>	<i>S. mansoni</i>
<i>S. haematobium</i>	0.00		
<i>S. bovis</i>	7.78	0.00	
<i>S. mansoni</i>	8.25	12.12	0.00
B	<i>S. haematobium</i>	<i>S. bovis</i>	<i>S. mansoni</i>
<i>S. haematobium</i>	0.00		
<i>S. bovis</i>	2.29	0.00	
<i>S. mansoni</i>	3.69	2.86	0.00

**Table 5**

*Schistosoma bovis* eggs published descriptions. EL, Egg Length; EW, Egg Width; BE50, Width at 50  $\mu$ m from blunt end; SL, Spine Length. Dash: unknown data.

Geographical Origin	Host	Sample	Technique	Size ( $\mu$ m) Mean $\pm$ Standard deviation (Minimum - Maximum)	N	Comments	Reference
Iraq	-	-	-	EL: 130 - 260	-		(MacHattie et al., 1933)
-	goat, sheep, cattle	faeces	Measurements were taken with a stage micrometre and a camera lucida, the eggs being drawn and afterwards measured with a rod calibrated against a standard magnification and micrometre	EL: 208 (179 - 232) EW: 55 (40 - 73) BE50: 31 (24-43)	500		(Alves, 1949)
Sudan	cattle	faeces	-	EL: 211 $\pm$ 14 (178 $\times$ 257) SL: 8.2 $\pm$ 3.0 (3.9 $\times$ 15.8) EL: 202	500	70% of spines not measurable	(Pitchford, 1965)
-	-	-	-	EW: 58	-		(Loker, 1983)
Kessounou Village, Ouémé, Benin	cattle	faeces	-	EL: 247.83 $\pm$ 4.03 (205 - 300) EW: 69.58 $\pm$ 1.56 (57.50-90) SL: 17.38 $\pm$ 0.68 (12.50-22.50) L/W Ratio: 3.59 $\pm$ 0.07 (2.93-4.17)	30 (20 for spine)		(Savassi et al., 2020)

**Table 6**

*Schistosoma haematobium* eggs published descriptions. EL, Egg Length; EW, Egg Width; BE50, Width at 50 µm from blunt end; SL, Spine Length. Dash: unknown data.

Geographical Origin	Host	Sample	Technique	Size (µm) Mean ± Standard deviation (Minimum -Maximum)	N	Comments	Reference
West Africa, the Sudan and Southern Rhodesia	Human	Urine	Measurements were taken with a stage micrometre and a camera lucida, the eggs being drawn and afterwards measured with a rod calibrated against a standard magnification and micrometre	EL: 142 (115-170) EW: 59 (46-80) BE50: 54 (44-74)	500		(Alves, 1949)
Malagasy (Madagascar)	Human	Urine	All microscopic work was done with wet preparations under a supported cover-slip. According to the author, it had been shown that the shape and measurements of eggs are not affected significantly by fixation in formol.	EL: 131 ± 11.6 (102-160) SL: 6.7 ± 2.4 (3.3-13.5)	150		(Pitcheford, 1965)
India				EL: 132 ± 11.8 (83-187) SL: 6.3 ± 2.4 (1.6-10.1)	200		
Egypt				EL: 131 ± 11.6 (108-162) SL: 6.4 ± 2.5 (1.9-19.7)	100		
Bechuanaland (Botswana)				EL: 139 ± 13.0 (95-174) SL: 7.3 ± 2.6 (1.9-15.8)	500		
Komatipoort, E. Transvaal (South Africa)				EL: 136 ± 13.6 (83-182) SL: 7.0 ± 2.6 (1.9-19.7)	1944		
E. Transvaal (South Africa)		faeces		Represented in a graph, no exact data			
		Bladder		Represented in a graph, no exact data			
	Rodents (various)			EL: 122 ± 11.8 (85-163) SL: 6.4 ± 2.7 (1.6-16.9)	980		
Ghana	Hamster	Liver and intestinal tissue	Liver and intestinal tissue was digested by the method of Smithers (1960) and the eggs laid by the parasites recovered. They were stored in 0.0% saline containing 125 IU penicillin per ml. Eggs were measured using a crossed micrometre eyepiece, either in utero in temporary mounts of adult females, or as suspensions of digested eggs. All eggs which were distorted, not lying horizontally, or partly obscured were rejected for purposes of measurements.	EL: 107.20 EW: 40.84 Length to max breadth: 60.94 SL: 7.91	116		(Jewsbury, 1968)
Nigeria				EL: 100.94 EW: 45.00 Length to max breadth: 52.37 SL: 5.69	16		
Shambat (Sudan)				EL: 102.54 EW: 39.78 Length to max breadth: 56.48 SL: 5.92	2238		
Cairo (Egypt)				EL: 99.12 EW: 36.73 Length to max breadth: 51.88 SL: 5.74	812		
Kisumu (Kenia)				EL: 92.28 EW: 36.93 Length to max breadth: 48.31 SL: 4.00	76		
Baghdad (Iraq)				EL: 114.29 EW: 44.27 Length to max breadth: 65.17 SL: 6.83	321		
Total				EL: 102.72 EW: 39.49 Length to max breadth: 56.17 SL: 5.98	3579		

Geographical Origin	Host	Sample	Technique	Size ( $\mu\text{m}$ ) Mean $\pm$ Standard deviation (Minimum -Maximum)	N	Comments	Reference
Shambat (Sudan)	Mice			EL: 88.21 EW: 34.00 Length to max breadth: 46.14 SL: 4.50	14		
Cahemba (Angola)				EL: 99.69 EW: 40.09 Length to max breadth: 52.70 SL: 5.44	270		
Cairo (Egypt)				EL: 94.30 EW: 37.10 Length to max breadth: 49.90 SL: 6.79	484		
Total				EL: 96.08 EW: 38.09 Length to max breadth: 50.82 SL: 6.27	768		
Shambat (Sudan)	<i>Arvicornhis</i>			EL: 91.64 EW: 35.74 Length to max breadth: 47.58 SL: 5.60	352		
	<i>Meriones</i>			EL: 85.12 EW: 33.84 Length to max breadth: 44.38 SL: 4.89	207		
	<i>Mastomys</i>			EL: 86.98 EW: 35.84 Length to max breadth: 44.09 SL: 6.17	58		
Ghana	Human	Urine	Eggs were measured using a crossed micrometre eyepiece, either in utero in temporary mounts of adult females, or as suspensions of digested eggs. All eggs which were distorted, not lying horizontally, or partly obscured were rejected for purposes of measurements.	EL: 135.59 EW: 59.09 Length to max breadth: 72.74 SL: 6.80	46		
Nigeria				EL: 135.02 EW: 58.36 Length to max breadth: 73.20 SL: 6.92	50		
Cairo (Egypt)				EL: 134.34 EW: 54.62 Length to max breadth: 69.21 SL: 7.07	95		
Dirdag (Yemen)				EL: 137.66 EW: 58.34 Length to max breadth: 70.89 SL: 7.09	79		
Said (Yemen)				EL: 122.92 EW: 54.95 Length to max breadth: 62.41 SL: 6.98	63		
Kuwait				EL: 118.15 EW: 45.15 Length to max breadth: 63.00 SL: 7.31	13		
Baghdad (Iraq)				EL: 125.99 EW: 59.82 Length to max breadth: 70.26 SL: 7.62	34		
Total				EL: 132.92 EW: 56.62 Length to max	380		

Geographical Origin	Host	Sample	Technique	Size ( $\mu\text{m}$ ) Mean $\pm$ Standard deviation (Minimum -Maximum)	N	Comments	Reference
Shambat (Sudan)	Mice			EL: 88.21 EW: 34.00 Length to max breadth: 46.14 SL: 4.50	14		
Cahemba (Angola)				EL: 99.69 EW: 40.09 Length to max breadth: 52.70 SL: 5.44	270		
Cairo (Egypt)				EL: 94.30 EW: 37.10 Length to max breadth: 49.90 SL: 6.79	484		
Total				EL: 96.08 EW: 38.09 Length to max breadth: 50.82 SL: 6.27	768		
Shambat (Sudan)	<i>Arvicornhis</i>			EL: 91.64 EW: 35.74 Length to max breadth: 47.58 SL: 5.60	352		
	<i>Meriones</i>			EL: 85.12 EW: 33.84 Length to max breadth: 44.38 SL: 4.89	207		
	<i>Mastomys</i>			EL: 86.98 EW: 35.84 Length to max breadth: 44.09 SL: 6.17	58		
Ghana	Human	Urine	Eggs were measured using a crossed micrometre eyepiece, either in utero in temporary mounts of adult females, or as suspensions of digested eggs. All eggs which were distorted, not lying horizontally, or partly obscured were rejected for purposes of measurements.	EL: 135.59 EW: 59.09 Length to max breadth: 72.74 SL: 6.80	46		
Nigeria				EL: 135.02 EW: 58.36 Length to max breadth: 73.20 SL: 6.92	50		
Cairo (Egypt)				EL: 134.34 EW: 54.62 Length to max breadth: 69.21 SL: 7.07	95		
Dirdag (Yemen)				EL: 137.66 EW: 58.34 Length to max breadth: 70.89 SL: 7.09	79		
Said (Yemen)				EL: 122.92 EW: 54.95 Length to max breadth: 62.41 SL: 6.98	63		
Kuwait				EL: 118.15 EW: 45.15 Length to max breadth: 63.00 SL: 7.31	13		
Baghdad (Iraq)				EL: 125.39 EW: 59.82 Length to max breadth: 70.26 SL: 7.62	34		
Total				EL: 132.92 EW: 56.62 Length to max	380		

Geographical Origin	Host	Sample	Technique	Size (µm) Mean ± Standard deviation (Minimum -Maximum)	N	Comments	Reference
Nigeria	Hamster	Tissues	-	breadth: 69.26 SL: 7.06 EL: 144.0 ± 14.2 EW: 58.4 ± 6.5	75		(Taylor, 1970)
Iran	-	-	-	EL: 135.4 ± 12.4 EW: 54.8 ± 6.5	75		
-	-	-	-	EL: 144 EW: 58	-		(Loker, 1983)
-	-	-	-	EL: 150 EW: 62	-		(Ross et al., 2002)
Doh (Republic of Benin)	Human	Urine	Mounted in saline solution (9% NaCl) beneath coverslips on glass slides	EL: 139±3 EW: 62±1	25	Based on morphology, might be hybrids	(Moaté et al., 2012)
Dangbo (Republic of Benin)	Human	Urine	Milli-Q water under a coverslip	EL: 146±1 EW: 61 ± 1	94		
Pakh, Northern Senegal	Human	Urine	Milli-Q water under a coverslip	EL: 136.5 ± 15.2 (92.4-176.4) EW: 61.8 ± 10.5 (35.7-93.0)	218	Mixed with hybrids	(Boon et al., 2017)
Pakh, Northern Senegal	-	Stool	-	EL: 136.9 ± 12.8 (110.4-173.2) EW: 63.9 ± 9.1 (42.0-86.5)	85		
-	-	-	-	EL: 83-187 EW: 60	-		(Candido et al., 2018)
Kessounou Village, Ouémé, Benin	cattle	faeces	The eggs were mounted individually at random in a 9% NaCl solution beneath glass coverslips on glass slides using a Pasteur pipette. They were photographed and measured by microscopy.	EL: 140 ± 42.43 (110 - 170) EW: 67.50 ± 17.68 (55-80) SL: 17.50 ± 3.54 (15-20) L/W Ratio: 2.06 ± 0.09 (2 -2.13)	2	For the <i>S. haematobium</i> morphotype, no major difference was observed between the origins (schoolchildren or cows)	(Savassi et al., 2020)
-	Human (schoolchildren)	Urine	-	EL: 138.85 ± 4.68 (120 - 175) EW: 68.85 ± 3.25 (50-95) SL: 10.31 ± 0.61 (7.50-12.50) L/W Ratio: 2.04 ± 0.07 (1.71 - 2.50)	13 (8 for spine)		
-	-	-	-	EL: 132.01 ± 1.66 (117.50 - 170) EW: 68.96 ± 1.62 (50-85) SL: 12.59 ± 0.48 (10-20) L/W Ratio: 1.94 ± 0.04 (1.63 - 2.60)	36 (29 for spine)		
-	-	-	-	EL: 110 - 170 EW: 438 - 70	-		(Zelbig, 2012)
-	-	-	-	EL: 150 EW: 62	-		(Othman and El Ridi, 2014)
-	-	-	-	EL: 144 EW: 58	-		(Lewis and Tucker, 2014)
-	-	-	-	EL: 150 EW: 50	-		(Mehlborn, 2016)
-	-	-	-	EL: 140 EW: 40	-		(Jain and Jain, 2019)
-	-	-	-	EL: 140 EW: 60	-		(LaVerde, 2019)
-	-	-	-	EL: 143 (112-170) EW: 60 (40-70)	-		(CDC, 2019)
-	-	-	-	EL: 110-170 EW: 40-70	-		(The Ohio State University,)
-	-	-	-	EL: 110-170 EW: 40-70	-		(MCI International,)
-	-	-	-	EL: 110-170 EW: 40-70	-		(The Australian Society for Parasitology Inc.,)
-	-	-	-	EL: 110 -170	-		(WHO, 2019)
-	-	-	-	EL: 144 EW: 58	-		(IARC, 2018)

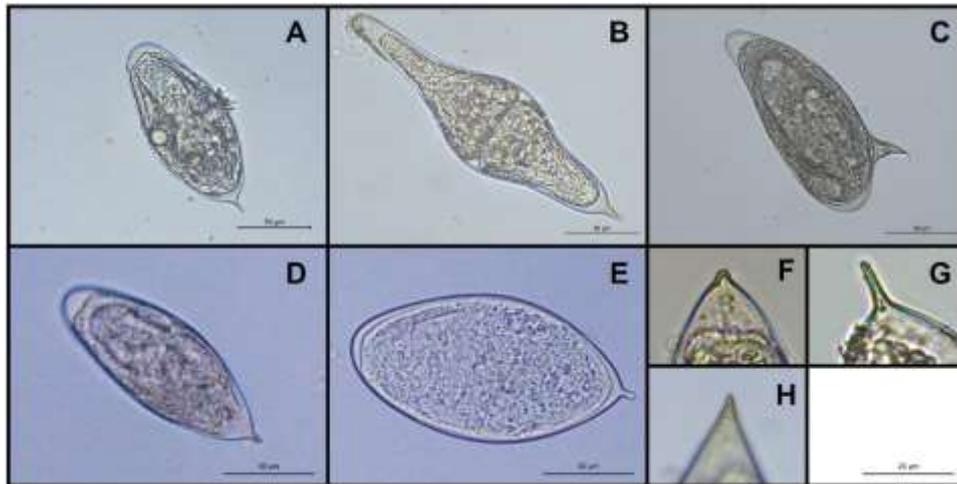
**Table 7**

*Schistosoma mansoni* eggs published descriptions. EL, Egg Length; EW, Egg Width; BE50, Width at 50  $\mu\text{m}$  from blunt end; SL, Spine Length. Dash: unknown data.

Geographical Origin	Host	Sample	Technique	Size ( $\mu\text{m}$ ) Mean $\pm$ Standard deviation (Minimum -Maximum)	N	Comments	Reference
-	-	-	-	EL: 142 EW: 60	-	-	(Loker, 1983)
-	Mice	Intestine	On each day of incubation, eggs laid in vitro were collected and mounted on regular flat slides using solid Vaseline as a spacer. For comparison between egg growth under in vitro and in vivo conditions, isolated eggs from the murine host were also analysed.	-	30	The area A of each egg was estimated by considering its longest length (major axis) and its longest width (minor axis), in accordance to the area formula of an ellipse: $A = (\pi \times \text{major axis} \times \text{minor axis})/4$ .	(Jurberg et al., 2009)
-	-	-	-	EL: 112-175 EW: 45-70	-	-	(Candido et al., 2015)
-	-	-	-	EL: 117 - 150 EW: 40-70	-	-	(de Souza et al., 2019)
Belo Horizonte, Minas Gerais, Brazil (BH strains)	mice	faeces	Measured using Image Pro-Lite	EL: 139.34 EW: 56.89	31	The length and width of the SD-strain eggs were significantly longer than the eggs from other strains and the eggs of the BH and SJ strains did not differ in length. Eggs from the BH strain had the shortest width. The size	(Izazobio et al., 2012)
Vale do Rio Paraíba, São Paulo, Brazil (SJ strains)	-	-	-	SL: 21.22 EL: 141.11 EW: 59.37	31	of the spicule was not significantly different amongst the strains; however, some eggs from the SD strain had spicules with a very curved tip.	
Jardim São Domingos, Campinas, São Paulo, Brazil (SD strains)	-	-	-	SL: 21.09 EL: 155.60 EW: 64.63 SL: 20.85	23		
-	-	-	-	EL: 150 EW: 40	-	-	(Lenzi et al., 2008)
-	-	-	-	EL: 140 EW: 61	-	-	(Othman and El Ridi, 2014)
-	-	-	-	EL: 140 EW: 60	-	-	(Lewis and Tucker, 2014)
-	-	-	-	EL: 140 (114-180) EW: 66 (45-73)	-	-	(CDC, 2019)
-	-	-	-	EL: 150	-	-	(Michigan State University, 1999)
-	-	-	-	EL: 115-175 EW: 45-70	-	-	(The Australian Society for Parasitology Inc.,)
Côte d'Ivoire	Human	Urine	Microscopic examination at 100x and 400x magnifications	EL: $150 \pm 3.20$ (146 - 156) EW: $62.5 \pm 1.73$ (60-66) SL: $25 \pm 1.35$ (23.5-27.5)	10		(Depaquit et al., 2019)

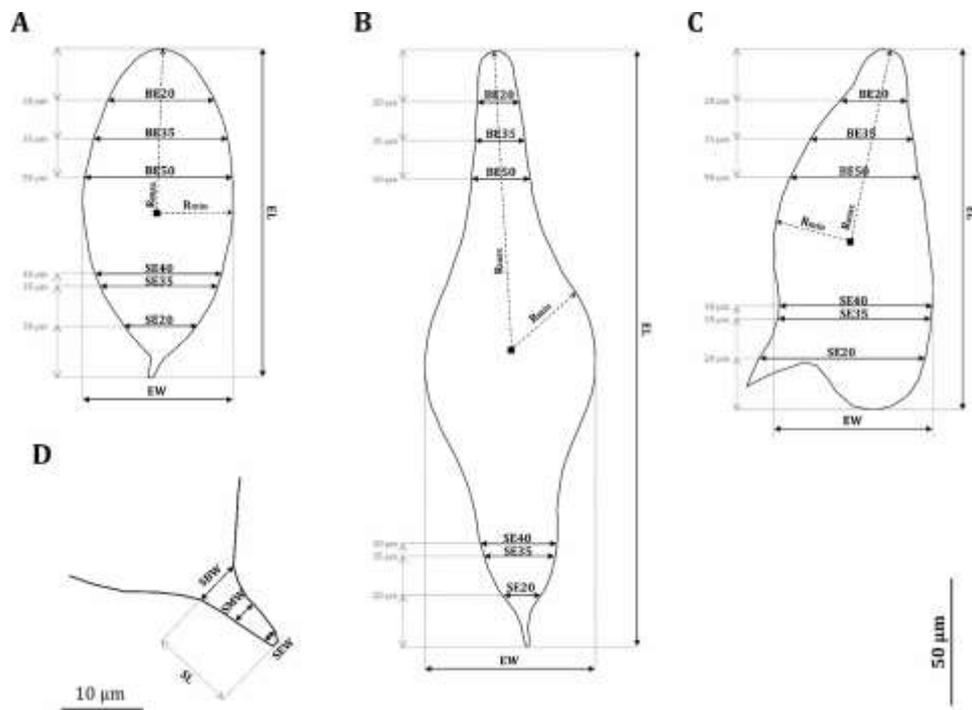
**Figure 1**

Egg sample of each *Schistosoma* species without coverslip: (A) *S. haematobium*, (B) *S. bovis*, (C) *S. mansoni* and comparison of one *S. haematobium* egg (D) without and (E) with coverslip. (F, G, H) Great variability in the *S. haematobium* spines was observed (optical microscopy, original magnification, A–E = $\times$ 400; no stain used). Scale bars: A–E = 50  $\mu$ m; F–H = 25  $\mu$ m.



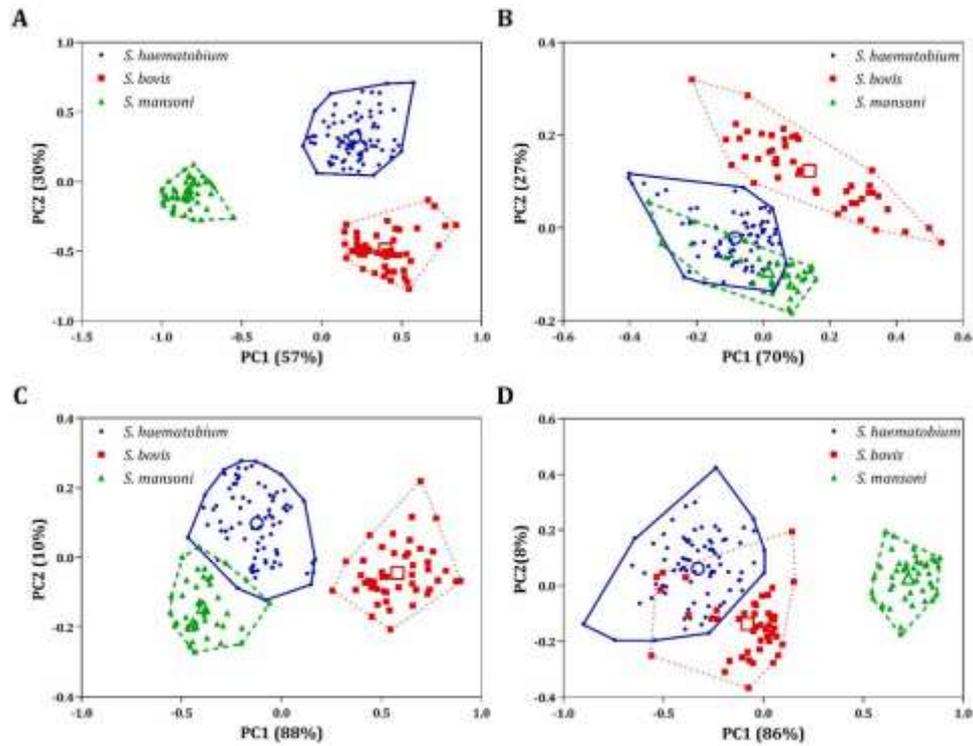
**Figure 2**

Standardised measurements designed for the morphometric phenotyping of the eggs of *Schistosoma* species.



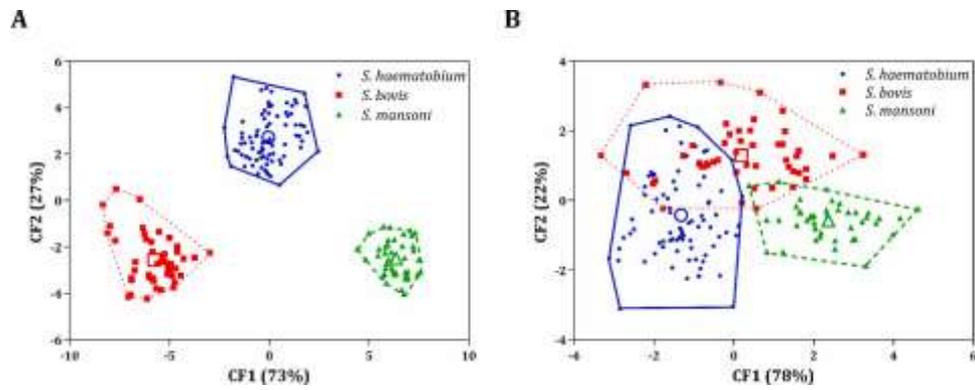
### Figure 3

Principal component analysis factor map of A) all measurements (17), B) size measurements (6), C) shape measurements (7) and D) spine measurements (4) of the three *Schistosoma* species analysed (*S. haematobium*, *S. bovis* and *S. mansoni*). Larger symbols represent the group centroid. In brackets, PC contribution.



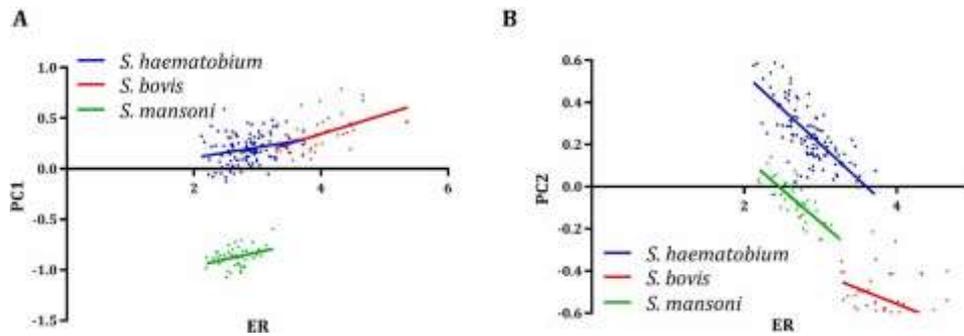
**Figure 4**

Discriminant analysis factor map of A) all measurements (17) and B) spine measurements (4) of the three Schistosomaspecies analysed (*S. haematobium*, *S. bovis* and *S. mansoni*). Larger symbols represent the group centroid. In brackets, canonical factor (CF) contribution.



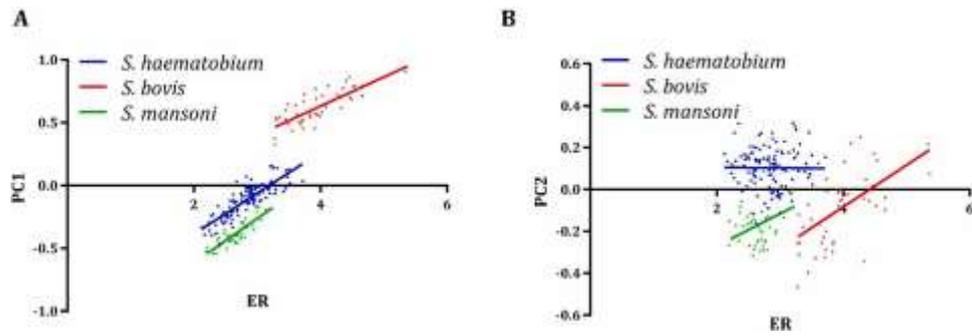
**Figure 5**

Relationship between elongation ratio (ER) values (maximum ratio divided by minimum ratio) and A) PC1 scores (57% contribution) and B) PC2 scores (30% contribution) of size, shape and spine measurements. On PC1, increasingly elongated eggs are associated with increasingly significant higher scores for the three *Schistosoma* species. On PC2, increasingly elongated eggs are associated with decreasingly significant lower scores for the three *Schistosoma* species. rs: r square, Y: orthogonal regression line.



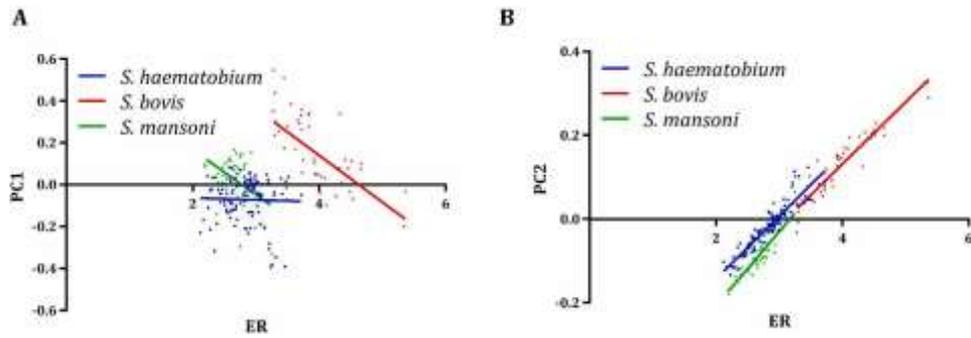
### Figure 6

Relationship between elongation ratio (ER) values (maximum ratio divided by minimum ratio) and A) PC1 scores (77% contribution) and B) PC2 scores (14% contribution) of size and shape measurements. On PC1, increasingly elongated eggs are associated with increasingly significant higher scores for the three *Schistosoma* species. On PC2, increasingly elongated eggs are associated with increasingly higher scores for *S. bovis* and *S. mansoni*, while no significant correlation was detected for *S. haematobium*. rs: r square, Y: orthogonal regression line.



**Figure 7**

Relationship between elongation ratio (ER) values (maximum ratio divided by minimum ratio) and A) PC1 scores (70% contribution) and B) PC2 scores (27% contribution) of size measurements. On PC1, increasingly elongated eggs are associated with increasingly lower scores for *S. bovis* and *S. mansoni*, while no correlation was detected for *S. haematobium*. On PC2, increasingly elongated eggs are associated with increasingly higher scores for all species. rs: r square, Y: orthogonal regression line.



### Figure 8

Relationship between elongation ratio (ER) values (maximum ratio divided by minimum ratio) and A) PC1 scores (88% contribution) and B) PC2 scores (10% contribution) of shape measurements. On PC1, increasingly elongated eggs are associated with increasingly higher scores for all species. On PC2, negative significant correlation was only detected for *S. haematobium* eggs. rs: r square, Y: orthogonal regression line.

