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# Cattle as natural host for Schistosoma haematobium (Bilharz, 1852) Weinland, 1858 x Schistosoma bovis Sonsino, 1876 interactions, with new cercarial emergence and genetic patterns — Source link

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HELMINTHOLOGY - ORIGINAL PAPER

# Cattle as natural host for *Schistosoma haematobium* (Bilharz, 1852) Weinland, 1858 x *Schistosoma bovis* Sonsino, 1876 interactions, with new cercarial emergence and genetic patterns

Boris A.E.S. Savassi[1,2] · Gabriel Mouahid[1] · Chrystelle Lasica[1] · Samoussou-Dine K. Mahaman[2] · André Garcia[3] · David Courtin[4] · Jean-François Allienne[1] · Moudachirou Ibikounlé[2] · Hélène Moné[1]

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**Abstract**
Schistosomiasis remains a parasitic infection which poses serious public health consequences around the world, particularly on the African continent where cases of introgression/hybridization between human and cattle schistosomiasis are being discovered on a more frequent basis in humans, specifically between *Schistosoma haematobium* and *S. bovis*. The aim of this paper is to analyze the occurrence of *S. bovis* in cattle and its relationship with *S. haematobium* in an area where cattle and humans share the same site in Benin (West Africa). We used the chronobiology of cercarial emergence as an ecological parameter and both molecular biology (COI mtDNA and ITS rDNA) of the larvae and morphology of the eggs as taxonomic parameters. The results showed a chronobiological polymorphism in the cercarial emergence rhythm. They showed for the first time the presence of *S. bovis* in Benin, the presence of introgressive hybridization between *S. bovis* and *S. haematobium* in domestic cattle, and the presence of atypical chronobiological patterns in schistosomes from cattle, with typical *S. haematobium* shedding pattern, double-peak patterns, and nocturnal patterns. Our results showed that the chronobiological life-history trait is useful for the detection of new hosts and also may reveal the possible presence of introgressive hybridization in schistosomes. Our results, for the first time, place cattle as reservoir host for *S. haematobium* and *S. bovis x S. haematobium*. The consequences of these results on the epidemiology of the disease, the transmission to humans, and the control of the disease are very important.

**Keywords** *Schistosoma bovis* · *Schistosoma haematobium* · Introgressive hybridization · Cercarial emergence pattern · Cattle · Benin

## Introduction

Schistosomiasis or Bilharziasis remains a parasitic infection which poses serious public health consequences around the world, particularly on the African continent which harbors 85% of the total number of infected people worldwide (Chitsulo et al. 2000; Engels et al. 2002). In Africa, humans can be infected with four species of *Schistosoma*: *Schistosoma mansoni* Sambon, 1907, *S. haematobium*, *S. intercalatum* Fisher, 1934 and *S. guineensis* Pagès, Jourdane, Southgate & Tchuem Tchuenté, 2003 where *S. haematobium* is the most prevalent species affecting around 112 million people (WHO 2019). Cattle schistosomiasis affects approximately 165 million domestic cattle worldwide and the disease is of veterinary and economic significances (De Bont and Vercruysse 1997). In Africa, species such as *S. bovis*, *S. curassoni* Brumpt, 1931, *S. mattheei* Veglia & Le Roux 1929, and *S. leiperi* Le Roux, 1955 are responsible for inducing severe infections in animals (De Bont and Vercruysse 1998). Africa is also the continent where cases of introgression/hybridization between human and cattle schistosomes are being discovered on a more frequent basis, specifically between *S. haematobium* and

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*S. bovis*. All but one of these cases were found in humans in West Africa: in Niger (introgression of *S. haematobium* by genes of *S. bovis*, Brémond et al. 1993; Leger et al. 2016), in Senegal (bidirectional introgression, Huyse et al. 2009; Webster et al. 2013), in Mali (hybrids, Soentjens et al. 2016), in Côte d'Ivoire (Angora et al. 2019), and in Benin (introgressive hybridization, Moné et al. 2015). The outlying case was from Corsica (France) (introgressive hybridization, Moné et al. 2015). Hybridization between *S. haematobium* and *S. bovis* in a non-human host was found in one rodent, *Mastomys huberti* Wroughton, 1909 in Senegal, which was shown to harbor a hybrid female (Catalano et al. 2018).

In Benin, two species of human schistosomes are reported in the literature: *S. haematobium* and *S. mansoni* (Moné et al. 2010): *S. haematobium* is widely distributed, with a national prevalence of 17.6%, while *S. mansoni* has a narrow distribution, with a prevalence of 2.5% (Onzo-Aboki et al. 2019). Introgressive hybridization between *S. bovis* and *S. haematobium* was already reported for humans in Benin (Moné et al. 2015) but, as a species, *S. bovis* was never reported in this country. This is interesting given that all of Benin's neighboring countries including Togo, Burkina Faso, Niger, and Nigeria were known to harbor this bovine species (Moné et al. 1999). In many villages in Benin, humans and cattle live in close proximity. Since the snails from the genus *Bulinus* are known to act as intermediate hosts for both *S. haematobium* and *S. bovis*, a logical question to consider is whether cattle in these areas could be infected by *S. bovis* or *S. haematobium* and if introgressive hybridization exists between the two species.

The aim of this paper is to analyze the occurrence of *S. bovis* in cattle and its relationship with *S. haematobium* in an area where cattle and humans share the same site (Fig. 1). We used the chronobiology of cercarial emergence as an ecological parameter, as the cercarial emission is related to the definitive host behavior (Théron et al. 1997; Ibikounlé et al. 2012; Mouahid et al. 2012; Mintsa-Nguema et al. 2014), the emission pattern of *S. bovis* cercariae is different (early diurnal, Mouahid et al. 1991) from that of *S. haematobium* cercariae (midday to late diurnal, Mouahid et al. 1991), and since schistosome cercarial emission has been shown to have a genetic base (Théron and Combes 1988). We also used both molecular biology (cytochrome *c* oxidase (COI mtDNA) and internal transcribed spacer (ITS rDNA)) of the larvae and morphology of the eggs as taxonomic parameters.

## Materials and methods

### Geographical sampling position

The study was conducted on cows and schoolchildren (elementary school; latitude N: 6°34′39.84″; longitude E: 2°31′9.03″) from 2017 to 2018 in Kessounou Village, located in the department of Ouémé in southeastern Benin (Fig. 2). The district of Kessounou is a fluvio-lacustrine zone characterized by a four-season subequatorial climate (Abou et al. 2018): (i) a long rainy season from March to June, (ii) a short dry season in July and August, (iii) a short rainy season in September and October, and finally (iv) a long dry season that runs from November to February. The Ouémé River crosses Kessounou and periodically floods the entire area during its flood period (mid-July to early November). During the period where river water levels are low (December to June), almost all of the water disappears giving way to ponds in various places. The climatic characteristics observed at Kessounou favor the seasonal installation of freshwater snails, especially during the flood period. Adults engage in fishing, agriculture, and livestock breeding. Schoolchildren regularly fish and search for crabs and shrimps for sale, which places them in permanent contact with contaminated waters. Cattle breeding is done in collectivity. Cows are left to wander even during periods of flooding and they never leave Kessounou unless they are sold. According to the breeders, most of the cows were born in Kessounou and some have spent more than 20 years in the one location.

### *Schistosoma* egg and miracidium recoveries from cattle

The feces from 48 cows were collected individually (from 150 to 250 g), immediately after defecation. The infection status of each fecal sample was analyzed using the miracidial hatching test from 50 g of feces. The filtration technique consisted of placing the 50 g of feces in a container with half a liter of NaCl 9‰ saline solution. The solution was then homogenized using an electric hand mixer, passed through a series of decreasing mesh vacuum sieves (315 μm, 180 μm, 106 μm, and 45 μm), then washed with NaCl 9‰ saline solution using a pressure pump. The residues retained on the 45 μm pore size sieve were placed in a beaker containing drilling water, at room temperature under light, for miracidial hatching. Among the 48 cows, 35 were found positive (73%). We chose to continue the analyses with the four fecal samples that gave the most miracidia (BK19, BK24, BK26, and BK29). The rest of the fecal sample from each of the four cows was then filtered as above and separated into two subsamples: the first subsample was once again passed through the 45 μm pore size sieve, and the eggs were placed in a beaker containing drilling water for miracidial hatching. Several miracidia from each cow were collected individually onto FTA® Classic Cards (Whatman, GE Healthcare companies, Little Chalfont, United Kingdom) and then transferred to University of Perpignan (France) for molecular analyses; the rest of the miracidia from the four cows were mixed and used for snail infection; the second subsamples from the four cows were once again individually

**Fig. 1** Kessounou sampling area where cows access water in close proximity to houses

passed through the 45 μm pore size sieve in 9‰ NaCl saline solution and the eggs from the four subsamples were mixed for an egg morphology study.

### *Schistosoma* egg and miracidium recoveries from schoolchildren

Urine samples from 76 schoolchildren were collected individually. The infection status of each urine sample was analyzed using the syringe filtration technique (Boko et al. 2016; Onzo-Aboki et al. 2019) after staining each filter with Lugol solution. One of the 18 girls' samples (5.6%) and 4 of the 58 boys (6.9%) were found positive. The rest of the five positive urine-egg samples (DK20, DK23, DK54, KE14, and KE15) were individually filtered according to the same procedure as above, and the filters were placed in saline solution. Two subsamples were made from each urine sample: the first subsample was passed through a 45 μm pore size sieve and the eggs were placed in a beaker containing drilling water for miracidial hatching. Several miracidia were collected individually for each urine sample in Eppendorf tubes (only one miracidium per tube), containing 95° alcohol, then transferred to the University of Perpignan (France) for molecular analyses. The second subsamples from the five urine samples were mixed in saline solution for an egg morphology study.

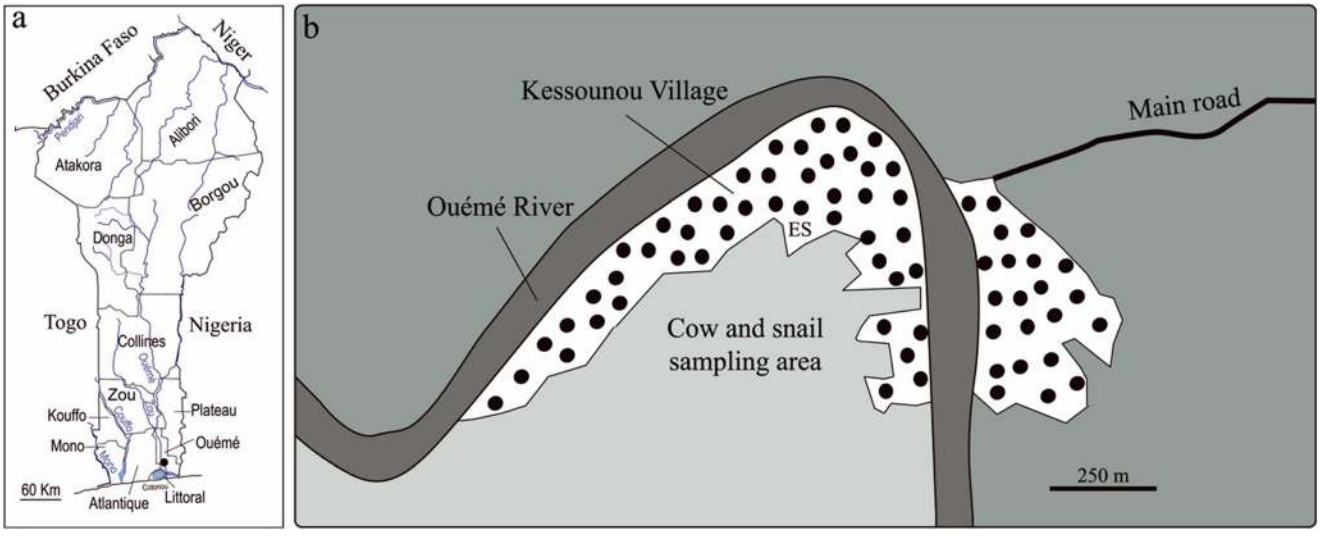


**Fig. 2** Sampling area. **a** Map of Benin; Kessounou Village (black circle) in Ouémé Department. **b** Kessounou Village (white with black circles). ES: Elementary School

The stools from the four urine-egg-positive boys were collected individually. Each stool was passed through a series of sieves (315, 180, 106, and 45 pore size) and washed through with 9‰ NaCl saline solution with a pressure pump.

The eggs that were retained on the 45 μm pore size sieve were collected in a beaker containing saline solution. The presence of eggs was ascertained under a binocular microscope. Only one stool was found positive and two subsamples were made: the first subsample was passed through the 45 μm pore size sieve and the eggs were placed in a beaker containing drilling water for miracidial hatching. Several miracidia were collected individually in Eppendorf tubes (only one miracidium per tube) containing 95° alcohol, then transferred to the University of Perpignan (France) for molecular analyses. The second subsample was kept in 9‰ NaCl saline solution for an egg morphology study.

## Snail infection

Two species of freshwater snails, *Bulinus forskalii* (Ehrenberg, 1831) and *Bulinus globosus* (Morelet, 1866), were collected in Kessounou Village and used in this study. These species were identified using shell morphology (Brown 1994). All of the collected snails were tested for infection with schistosomes or other trematodes for a period of 55 days. None of the snails used for the infection were found to be naturally infected. We exposed 43 *Bulinus forskalii* (6.3 to 8.2 mm in height) and 62 *Bulinus globosus* (4.4 to 7.6 mm in height), individually, to 3 miracidia obtained from the eggs originating from a mixture of feces from the 4 cows (BK19, BK24, BK26, and BK29). The snails of each species were maintained separately, fed with dry and fresh salad in water at 28 °C with a balanced photoperiod (12 h light, 12 h dark) and a photophase from 6:00 am to 6:00 pm. The snails were then transferred to Perpignan (France) for cercarial emission and molecular analyses.

## Cercarial emergence pattern

The snails were maintained individually in glass vessels containing 150 ml of drilling water, at a constant temperature (25 °C), balanced photoperiod (light/dark: 12 h/12 h), and photophase (6:00 am to 6:00 pm). They were given fresh washed lettuce ad libitum. The cercarial emission rhythm (chronobiology) was studied for five consecutive days for 13 infected snails, 11 *Bulinus forskalii* (Kf1 to Kf11), and 2 *Bulinus globosus* (Kg1 and Kg2) to show the stability of the emission pattern, from the 6th day after the first day of the patent period, i.e., 32 to 38 days after snail exposure to the miracidia. Each hour, each snail, with its lettuce, was transferred into a new vessel. The water left in the vessel, containing the cercariae, was transferred to a container where a few drops of Lugol solution was added to kill the cercariae and red-color them; this solution was then filtered through a Nitrel polyamide filter (25 μm pore size). The cercariae retained on the filter were counted under the binocular microscope.

Cercariae emitted by four infected *Bulinus forskalii* (Kf5, Kf6, Kf8, and Kf9) and the two *Bulinus globosus* (Kg1 and Kg2) were individually stored onto FTA® Classic Cards (Whatman, GE Healthcare companies, Little Chalfont, United Kingdom), as described above, for molecular studies.

## Molecular analyses

### DNA extraction

DNA from miracidia collected in alcohol was extracted according Beltran et al. (2008) and stored at − 20 °C. DNA from either miracidia or cercariae individually stored on FTA® Classic Cards (Whatman, GE Healthcare companies, Little Chalfont, United Kingdom) was extracted as follows. A 3-mm disk was removed with a Craft Punch from the FTA® Classic Cards at the center of where the miracidium or cercaria was loaded. The disks were deposited in Eppendorf tubes (1.5 ml) and an initial washing step with 100 μl of Milli-Q water was performed. After a 10-mn incubation at room temperature, water was removed and replaced with 80 μl of 5% Chelex® 100 Molecular Biology Grade Resin solution (Bio-Rad Laboratories, Hercules, California, USA). The sample was then heated to 65 °C for 30 min at a stirring speed of 800 rpm followed by a second heating at 99 °C for 8 min without stirring. DNA was finally collected by centrifugation at 14,000 rpm for 2 min. Fifty microliters of the supernatant (DNA) of each sample was taken and stored at − 20 °C for further molecular analyses.

### Rapid diagnostic multiplex PCR

A rapid diagnostic multiplex PCR was used to target the COI mtDNA gene. We used the technique of Webster et al. (2010), optimized by Van den Broeck et al. (2011) and Angora et al. (2019). The primers were one universal reverse primer (Shmb.R: 5′-CAA GTA TCA TGA AAY ART ATR TCT AA-3′) and three species-specific forward primers (for *S. haematobium* (120 bp) Sh.F: 5′-GGT CTC GTG TAT GAG ATC CTA TAG TTT G-3′, for *S. bovis* (260 bp) Sb.F: 5′-GTT TAG GTA GTG TAG TTT GGG CTC AC-3′ and for *S. mansoni* (215 bp) Sm.F: 5′-CTT TGA TTC GTT AAC TGG AGT G-3′). Each PCR amplification of partial COI mtDNA was carried out in a total volume of 10 μl, containing 2 μl of DNA template, 2 μl of the GoTaq® Flexy buffer (Promega; Madison, Wisconsin, USA), 0.6 μl of 25 mM $MgCl_2$, 1 μl of 10× primer mix (4 μl of 100 μM universal reverse primer, 4 μl of each 100 μM forward primer, and 84 μl of milli-Q water), 0.2 μl solutions of dNTP at 10 mM each, 0.2 μl of the GoTaq® G2 Hot Start Taq Polymerase

(Promega), and 4 μl milli-Q water. The PCR conditions involved an initial phase of activation of GoTaq Polymerase at 95 °C for 3 min followed by 45 amplification cycles. Each cycle comprised DNA denaturation step at 95 °C for 10 s, followed by primer annealing at 52 °C for 30 s, and an elongation step at 72 °C for 10 s. The program ended with a final extension at 72 °C for 2 min. The PCR products were examined on 2.5% agarose gels at 135 V for 40 min using the 100 bp DNA size marker (Promega) for size estimation.

### COI mtDNA PCR

Partial COI mtDNA amplification was performed by PCR using the forward primer, Cox1_schist F: 5′-TCTT TRGATCATAAGCG-3′, and the reverse primer, Cox1_schist-R: 5′-TAATGCATMGGAAAAAAACA-3′ (Lockyer et al. 2003). PCR conditions used were those as described in Moné et al. (2015).

### ITS rDNA PCR

PCR amplification of ITS rDNA (partial 18S, ITS1, 5.8S, and ITS2) was performed using the primers of Barber et al. (2000): ITS4F: 5′-TCCTCCGCTTATTGATATGC-3′; ITS5R: 5′-GGAAGTAAAAGTCGTAACAAGG-3′. The amplification was carried out in a total reaction volume of 25 μl, containing 2 μl of DNA; 1.5 μl of $MgCl_2$ (25 mM), 5 μl of the 5× buffer (Promega), 1 μl of each of the primers (10 μM), 0.5 μl of each dNTP (10 mM), 0.2 μl of Go Taq polymerase (Promega), and 13.8 μl of H2O using a thermocycler (Techne TC-Plus, Bibby Scientific, Staffordshire, UK). The reaction conditions included an activation step at 95 °C for 3 min, followed by 45 amplification cycles. Each cycle comprised a DNA denaturation step at 95 °C for 40 s, followed by primer annealing at 48 °C for 40 s and an elongation step at 72 °C for 1 min 10 s and a final extension at 72 °C for 5 min. One percent agarose gel electrophoresis was used to visualize the ITS rDNA-PCR products.

### Sequencing

Partial COI mtDNA gene and ITS rDNA region were sequenced (Genoscreen; Lille, France) using the reverse primers; some regions were also sequenced using the forward primer in order to confirm the sequence. The sequences were manually edited using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, USA). For the analysis of nuclear gene sequences (ITS rDNA), the sequence polymorphism was verified and confirmed by visualization of the raw sequence chromatograms. Since this gene has biparental transmission and segregation sites (polymorphic sites) between *S. haematobium* and *S. bovis*, the sequence chromatograms were carefully checked to identify the presence of possible heterozygosity. At each polymorphic site where two chromatogram peaks overlapped (indicating the genetic signature of both parents), the IUPAC ambiguity symbols were used to indicate the individual nucleotide polymorphisms. Thus, Y indicates the presence of the bases T and C, rather than an ambiguous reading between T and C. Similarly, R indicates the presence of the bases A and G and not an ambiguous reading between A and G. The different haplotypes and the different profiles are presented in Table 1 for the COI mtDNA gene and the ITS rDNA region, respectively.

### Partial COI mtDNA phylogenetic analysis

DNA multiple sequence alignments were performed using Muscle program (Edgar 2004) on the MEGA 7.0 software (Kumar et al. 2016) and refined by Gblocks 0.91b (Castresana 2000; Dereeper et al. 2008, 2010). The probabilistic model of sequence evolution (Nei and Kumar 2000) and the gamma distribution (G) to approximate rate heterogeneity among haplotypes was performed using MEGA 7.0 software. The best model with the lowest BIC score (Bayesian Information Criterion) was HKY + G (Hasegawa-Kishino-Yano with the gamma distribution). A phylogenetic tree using the maximum likelihood method was constructed. The maximum likelihood method was performed using MEGA 7.0 software. Reliability for internal branch was assessed using bootstrapping procedure (1000 replicates). The topology of the tree was rooted by *S. intercalatum*. The comparison was also performed using the percentage of difference calculated from the pairwise distances data obtained by using MEGA 7.0 software.

## Egg morphology

The eggs were mounted individually at random in a 9‰ NaCl solution beneath glass cover-slips on glass slides using a Pasteur pipette. They were photographed and measured (length, width, spine length, and length/width ratio) by microscopy. Only the eggs which contained a living miracidium were measured. In total, 113 eggs were measured: 47 from cow feces, 48 from schoolchildren urines, and 18 from the schoolchildren stool.

## Statistical analyses

Means and standard errors were calculated. The Mann-Whitney test was used for measure comparisons and the Fisher's exact test was used for proportion comparisons using the BiostaTGV https://biostatgv.sentiweb.fr. The probability values ($p$) less than 5% were considered statistically significant.

**Table 1** Origin and sample label of the miracidia and cercariae used for the molecular analyses. Number of haplotypes/number of sequenced larval stages (either miracidia or cercariae) for the COI mtDNA gene and number of profiles/number of sequenced larval stages (either miracidia or cercariae) for the ITS rDNA region

| Host_Biological material_Larval stage | Sample label | COI mtDNA | ITS rDNA |
|---|---|---|---|
| Cow_feces_miracidium | BK19 | 3/5 | 2/5 |
| | BK24 | 2/5 | 3/5 |
| | BK26 | 2/5 | 3/5 |
| | BK29 | 3/5 | 2/5 |
| *B. forskalii* exposed to miracidia from cows_Cercaria | Kf5 | 1/5 | 1/5 |
| | Kf6 | 1/3 | 1/3 |
| | Kf8 | 1/3 | 1/3 |
| | Kf9 | 2/3 | 1/3 |
| *B. globosus* exposed to miracidia from cows_Cercaria | Kg1 | 1/5 | 1/5 |
| | Kg2 | 1/5 | 1/5 |
| Child_urine_miracidium | DK20 | 1/3 | – |
| | DK23 | 4/6 | 5/6 |
| | DK54 | 2/2 | 1/2 |
| | KE14 | 2/11 | 6/14 |
| | KE15 | 4/11 | 5/10 |
| Child_stool_miracidium | KE14 | 3/14 | 7/13 |

BK, Kf, and Kg: cow origin; DK and KE: schoolchild origin; "–": not available

## Results

### Snail infection

Of the 43 *B. forskalii* that were exposed, 16 survived and 11 shed cercariae (69%) (Kf1 to Kf11); of the 62 *B. globosus* that were exposed, 52 survived and only 2 shed cercariae (2.9%) (Kg1 and Kg2). The percentage of infection was significantly higher for *B. forskalii* compared to *B. globosus* (Fisher's exact test; $p < 0.0001$). Prepatent periods lasted 26 days (from the *B. forskalii* Kf1, Kf2, Kf3, Kf4, Kf5, Kf6, Kf7, and Kf8 and from the *B. globosus* Kg1), 30 days (from the *B. forskalii* Kf9 and Kf10), or 32 days (from the *B. forskalii* Kf11 and from the *B. globosus* Kg2).

### Cercarial emission patterns

The cercarial emission patterns were analyzed for the 13 positive snails (11 *B. forskalii* and 2 *B. globosus*) during 5 consecutive days (65 occurrences in total) from the 6th day after the first day of the patent period, i.e. 32 to 38 days after snail exposure to the miracidia. The results showed a variability in the patterns; we identified four different patterns (Fig. 3), and each snail harbored only one pattern.

a- An early diurnal pattern was observed for 7 of the 13 snails (53.8%). Cercarial emission began at 6 am, immediately after the start of the light period; it increased very rapidly to reach an emission peak at either 7 am (for Kf1 and Kf11) or 8 am (for Kf2, Kf3, Kf6, Kf7, and Kf8), then decreased gradually to stop at 2 pm. Immediately after the beginning of the dark period, very low cercarial emissions were observed at 7 pm, and sometimes at 8 pm as well. The mean pattern showed that more than 97% of the cercariae were shed during the day and a peak occurred at 8 am (Fig. 3a);

b- A midday to late diurnal pattern was found for 1 of the 13 snails (7.7%). More than 90% of the cercariae were shed during the day. Cercarial emission mainly occurred from 12 am to 6 pm with an emission peak at 2 pm (Kg2), followed by a gradual decrease during the daylight hours and then a rapid decrease at the beginning of the dark period (Fig. 3b);

c- An early diurnal and nocturnal pattern was found for 4 of the 13 snails (30.8%). The percentages of cercariae shed during the day were highly variable and represented from more than 90% (as for the two previous chronotypes) to less than 60%. The diurnal cercarial emission occurred during the 4 to 5 first hours of light with peaks at 7 am (for Kf5), 8 am (for Kf4 and Kf9), or 9 am (for Kf10). The nocturnal cercarial emission occurred mainly during the first hour of darkness where another peak was observed for the 4 snails for which Kf5 represented more than 40% of the total cercarial daily emission. The mean pattern had more than 79% of the cercariae that were shed during the day and harbored two peaks, an early peak at 8 am and a nocturnal peak at 7 pm (Fig. 3c);

d- A late diurnal and nocturnal pattern for 1 of the 13 snails (7.7%). Roughly 60% of the cercariae were shed during

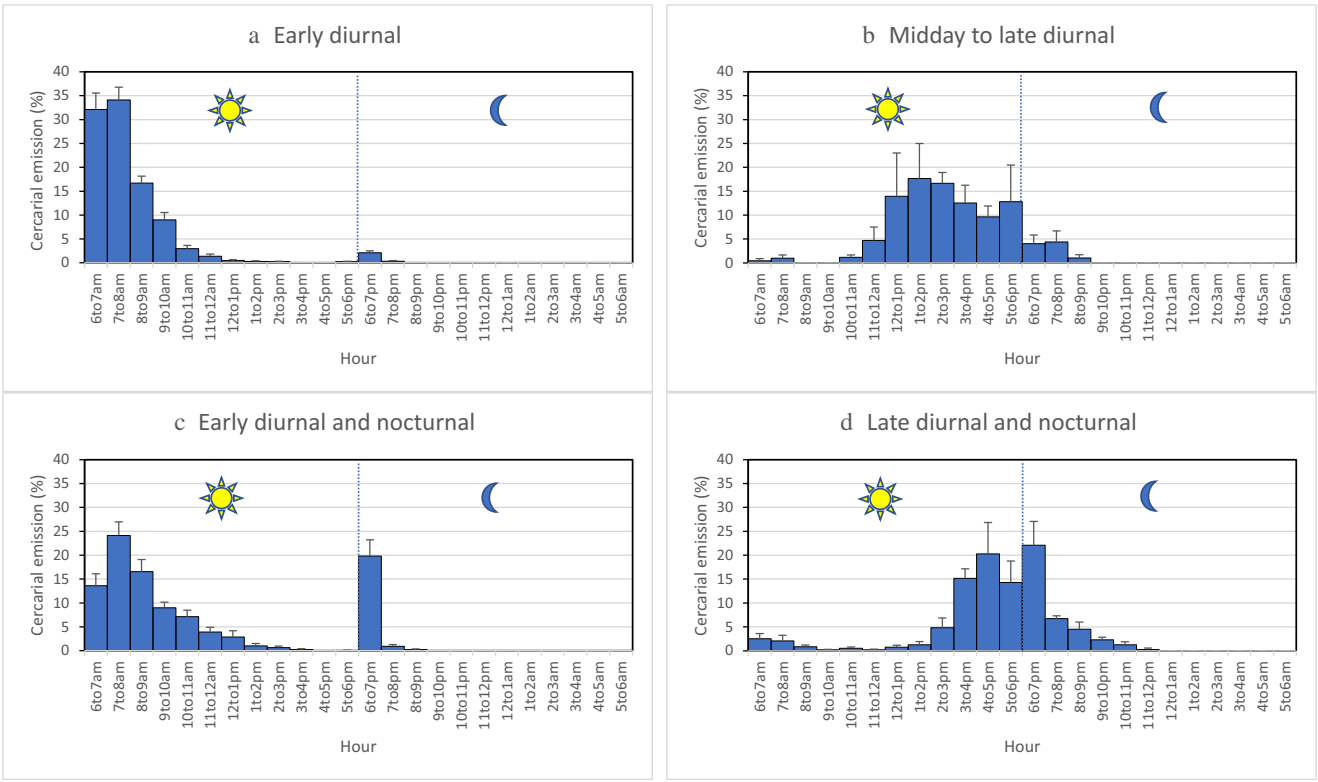


**Fig. 3** Cercarial emission patterns from infected snails, each exposed to 3 miracidia of *Schistosoma* from cow feces. **a** Early diurnal pattern for *B. forskalii* (Kf1, Kf2, Kf3, Kf6, Kf7, Kf8 and Kf11). **b** Midday to late diurnal pattern for *B. globosus* (Kg2). **c** Early diurnal and nocturnal pattern for *B. forskalii* (Kf4, Kf5, Kf9, Kf10). **d** Late diurnal and nocturnal pattern for *B. globosus* (Kg1)

the day. Cercarial emission occurred mainly from 4 pm to 7 pm with an emission peak at 7 pm (Kg1) (Fig. 3d).

## COI mtDNA (894 bp)

Ninety-one sequences were analyzed. The numbers of haplotypes on the numbers of sequenced larval stages for each sample (4 cows, 6 snails, and 5 schoolchildren) are presented in Table 1. We found from 2 to 3 haplotypes per cow, from 1 to 2 haplotypes per snail, and from 1 to 4 haplotypes per child. In total, 13 haplotypes were found: 6 haplotypes from cows (K_Cow_Hap1 to K_Cow_Hap6) which contained 13 mutation sites, all different from the 7 haplotypes from schoolchildren (K_Child_Hap7 to K_Child_Hap13), containing 16 mutation sites (Table 2). Sequence data were deposited in the NCBI GenBank database under the accession numbers MT159589 to MT159601. The percentages of differences ranged between 0.11 and 1.36% between the cow and the human haplotypes. Site polymorphism was shared between cows and schoolchildren in seven different positions while six positions were polymorph only in cows and only eight in schoolchildren. One haplotype was harbored by only one individual (either cow (K_Cow_Hap1: accession number MT159589, K_Cow_Hap2: accession number MT159590 and K_Cow_Hap6: accession number MT159594) or schoolchild (K_Child_Hap10: accession number MT159598, K_Child_Hap12: accession number MT159600 and K_Child_Hap13: accession number MT159601)) or was shared by different cows (K_Cow_Hap3: accession number MT159591, K_Cow_Hap4: accession number MT159592 and K_Cow_Hap5: accession number MT159593) or different schoolchildren (K_Child_Hap7: accession number MT159595, K_Child_Hap8: accession number MT159596, K_Child_Hap9: accession number MT159597 and K_Child_Hap11: accession number MT159599). Three different haplotypes were found from the snails among the six recovered in the cows; they were different between *B. forskalii* (K_Cow_Hap1: accession number MT159589 and K_Cow_Hap2: accession number MT159590) and *B. globosus* (K_Cow_Hap4: accession number MT159592). Two haplotypes (K_Child_Hap8: accession number MT159596 and K_Child_Hap9: accession number MT159597) were shared by miracidia that came from the same child in both the urine and the stool.

## ITS rDNA (946 bp)

Eighty-nine sequences were analyzed for the 18S, ITS1, 5.8S and ITS2 genes. The first 22 bp of our ITS rDNA sequences belonged to the 18S gene and no variability was found

**Table 2** The 13 haplotypes of COI mtDNA gene, harbored by cow (BK, Kf and Kg) and schoolchild (DK and KE) in Kessounou Village, Benin

| Host | Haplotype | 22 polymorphic positions (presented in vertical numbers) | | | | | | | | | | | | | | | | | | | | | | | Sample label (Number of sequenced larval stages) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 | 84 | 144 | 150 | 195 | 294 | 309 | 324 | 333 | 369 | 432 | 501 | 594 | 630 | 690 | 699 | 726 | 777 | 778 | 782 | 792 | 843 | | |
| Cow | K_Cow_Hap1 | A | A | G | A | C | G | A | G | T | G | C | G | A | T | T | T | G | T | T | C | G | T | | BK19(2); Kf9(2) |
| | K_Cow_Hap2 | . | . | A | . | T | A | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | C | | BK19(1); Kf5(5); Kf6(3); Kf8(3); Kf9(1) |
| | K_Cow_Hap3 | . | . | A | G | T | A | . | . | C | T | . | . | G | . | C | . | . | . | . | . | . | . | | BK19(2); BK26(1); BK29(2) |
| | K_Cow_Hap4 | . | . | A | G | T | A | . | . | C | T | . | . | . | . | C | C | . | C | . | . | . | . | | BK24(4); BK26(4); Kg1(5); Kg2(5) |
| | K_Cow_Hap5 | . | . | . | . | . | . | . | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | | BK24(1); BK29(2) |
| | K_Cow_Hap6 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | | BK29(1) |
| Child | K_Child_Hap7 | . | G | A | G | T | A | . | . | C | T | . | . | . | C | C | . | . | . | C | . | . | . | | DK20(3); DK23(1); KE15(1) |
| | K_Child_Hap8 | . | . | A | G | T | A | . | . | C | T | . | . | . | . | . | C | . | C | . | . | . | . | | DK23(3); DK54(1); KE14 (urine(3) and stool(2)) |
| | K_Child_Hap9 | . | . | A | G | T | A | . | . | C | T | T | . | . | . | C | C | . | C | . | . | . | . | | DK23(1); KE14 (urine(9) and stool(6)); KE15(8) |
| | K_Child_Hap10 | . | . | A | G | T | A | . | . | C | T | . | T | . | . | . | C | . | C | . | . | . | . | | DK23(1) |
| | K_Child_Hap11 | . | . | A | G | T | A | . | . | C | T | T | . | . | . | C | C | . | C | . | . | A | . | | DK54(1); KE14 (stool(5)) |
| | K_Child_Hap12 | . | . | A | . | T | A | G | . | . | . | . | . | . | . | . | . | A | . | . | . | . | C | | KE15(1) |
| | K_Child_Hap13 | G | . | A | . | T | A | G | . | . | . | . | . | . | . | . | . | A | . | . | . | . | C | | KE15(1) |

between any of our samples. We thus no longer gave this gene further consideration. Eleven profiles were found with the ITS1, 5.8S and ITS2 sequences, with 2 to 3 profiles per cow, only 1 profile per snail and 1 to 7 profiles per child (Table 1). Sequence data were deposited in the NCBI GenBank database under the accession numbers MT158872 to MT158882.

Table 3 shows that the 11 profiles may differ by their ITS1 gene (458 bp: from position 23 to position 480), with 5 genetic variants at three positions (TGT, TAT, TGY, TRT, and YAT), by their 5.8S gene (155 bp: from position 481 to position 635), with 2 genetic variants at 1 position (C and Y), and by their ITS2 gene (311 bp: from position 636 to position 946), with 3 genetic variants at 4 positions (ATAT, GCGC and RYRY). Among these 11 profiles, 1 profile corresponding to *S. bovis* ITS rDNA was found only in cows (profile 1: accession number MT158872), and 1 profile corresponding to *S. haematobium* ITS rDNA was found only in schoolchildren (profile 2: accession number MT158873). All of the other 9 profiles showed atypical sequences in ITS rDNA. Two were found only in cows (profiles 3: accession number MT158874 and 4: accession number MT158875) and showed double peaks in the ITS1 gene. Five were found only in schoolchildren (profiles 5: accession number MT158876, 6: accession number MT158877, 7: accession number MT158878, 8: accession number MT158879 and 9: accession number MT158880) and showed double peaks in 1 or 2 of the ITS1, 5.8S and ITS2 genes. Two were found both in cows and schoolchildren, where the first one showed a typical *S. haematobium* ITS1 marker together with a typical *S. bovis* ITS2 marker (profile 10: accession number MT158881) and the second showed a typical ITS1 *S. haematobium* marker together with double peaks in both the 5.8S and ITS2 genes (profile 11: accession number MT158882).

## COI mtDNA phylogeny (881 bp)

The 13 haplotypes obtained from the COI mtDNA sequences (haplotype code/COI mtDNA accession No: K_Cow_Hap1/MT159589, K_Cow_Hap2/MT159590, K_Cow_Hap3/MT159591, K_Cow_Hap4/MT159592, K_Cow_Hap5/MT159593, K_Cow_Hap6/MT159594, K_Child_Hap7/MT159595, K_Child_Hap8/MT159596, K_Child_Hap9/MT159597, K_Child_Hap10/MT159598, K_Child_Hap11/MT159599, K_Child_Hap12/MT159600, K_Child_Hap13/MT159601) were compared to 23 published nucleotide sequences of *Schistosoma* (12 sequences from *S. haematobium* (haplotype code/COI mtDNA accession No: Sh_Gambia/JQ397349; Sh_Liberia/JQ397350; Sh_Mali/AY157209; Sh_Egypt/JQ397368; Sh_Guinea Bissau/JQ397351; Sh_Toho_Benin/KT354661; Sh_Cameroon/JQ397365; Sh_Melen_Gabon/KT354660; Sh_Ekouk_Gabon/KT354659; Sh_Kenya/JQ397378; Sh_South Africa/JQ397397; Sh_Madagascar/JQ397399), 7 from *S. bovis* (haplotype code/COI mtDNA accession No: SbxSh_Corsica1_France/KT354656; SbxSh_Corsica2_France/KT354657; SbxSh_Corsica3_France/KT354658; SbxSh_Sô-Tchanhoué_Benin/KT354662; Sb_Senegal/AJ519521; Sb_Kenya/FJ897160; Sb_Tanzania/AY157212), 2 from *S. guineensis* (haplotype code/COI mtDNA accession No: Sg_Sao Tome and Principe/AJ519517 and Sg_Cameroon/AJ519522), 1 from *S. curassoni* (haplotype code/COI mtDNA accession No: Sc_ Senegal/AJ519516), and 1 from *S. intercalatum* (haplotype code/COI mtDNA accession No:

**Table 3** The 11 profiles of the ITS rDNA region, and their positions, harbored by cow (BK, Kf, and Kg) and schoolchild (DK and KE) in Kessounou Village, Benin

| Species | Profile | ITS1 | | | 5.8 | ITS2 | | | | Host | Sample (Number of sequenced larval stages) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 41 | 73 | 257 | 556 | 725 | 780 | 830 | 900 | | |
| *S. bovis* | 1 | T | G | T | C | A | T | A | T | Cow | BK19(4); BK24(1); BK26(1); BK29(4); Kf5(5); Kf6(3); Kf8(3); Kf9(3) |
| *S. haematobium* | 2 | T | A | T | C | G | C | G | C | Schoolchild | KE14 (urine(1) and stool(1)) |
| *S. bovis x S. haematobium* | 3 | T | G | Y | C | A | T | A | T | Cow | BK19(1); BK29(1) |
| | 4 | T | R | T | C | A | T | A | T | | BK24(3); BK26(3) |
| | 5 | T | A | T | Y | G | C | G | C | Schoolchild | DK54(2); KE14 (urine(4) and stool(2)); KE15(2) |
| | 6 | T | A | T | C | R | Y | R | Y | | DK23(1); KE14 (stool(1)); KE15(1) |
| | 7 | Y | A | T | C | G | C | G | C | | DK23(2); KE14 (urine(1) and stool(1)) |
| | 8 | Y | A | T | Y | G | C | G | C | | DK23(1); KE14 (urine(4) and stool(4)); KE15(4) |
| | 9 | Y | A | T | C | R | Y | R | Y | Cow and Schoolchild | DK23(1); KE14 (stool(2)); KE15(2) |
| | 10 | T | A | T | C | A | T | A | T | | BK24(1); BK26(1); Kg1(5); KE14 (urine(2)) |
| | 11 | T | A | T | Y | R | Y | R | Y | | Kg2(5); DK23(1); KE14 (urine(2) and stool(2)); KE15(1) |

Y indicates the presence of the bases T and C, rather than an ambiguous reading between T and C. Similarly, R indicates the presence of the bases A and G and not an ambiguous reading between A and G

Si_Democratic Republic of the Congo/AJ519515) obtained at the GenBank database. Maximum likelihood tree topology of COI mtDNA showed that all 13 haplotypes which came from cows and schoolchildren belonged to the *S. bovis* clade and not to the *S. haematobium* or to other *Schistosoma* species clades (Fig. 4). The percentages of differences ranged between 0.23 and 1.70% between our 13 haplotypes and the *S. bovis* haplotypes from Senegal, Kenya, and Tanzania. We found 0% difference between K_Child_Hap11 and the haplotype from Sô-Tchanhoué, Benin. We also found 0% difference between K_Child_Hap13 and the Corsica3 haplotype.

## Egg morphometry

Two or three morphotypes were observed according to the host and the excreta (urine or feces/stool). In cow feces, three morphotypes were found: *S. bovis*, *S. haematobium*, and their intermediate morphotype (Fig. 5a–c); in schoolchild stool (Fig. 5d, e) and urine (Fig. 5f, g), two morphotypes were found: *S. haematobium* and an intermediate morphotype.

The percentages of each morphotype are presented in Fig. 6 for each origin. The percentage of *S. bovis* morphotype was high (63.8%) in the cow feces, and null in schoolchild (stool and urine origins) ($p < 0.05$). The percentages of intermediate morphotype were not different between the origins ($p > 0.05$). The percentage of *S. haematobium* morphotype observed from cow feces origin was significantly lower than those from both stool and urine of schoolchild origins ($p < 0.05$). For each origin, the percentages of *S. bovis*, intermediate and *S. haematobium* morphotypes were all significantly different from each other ($p < 0.05$).

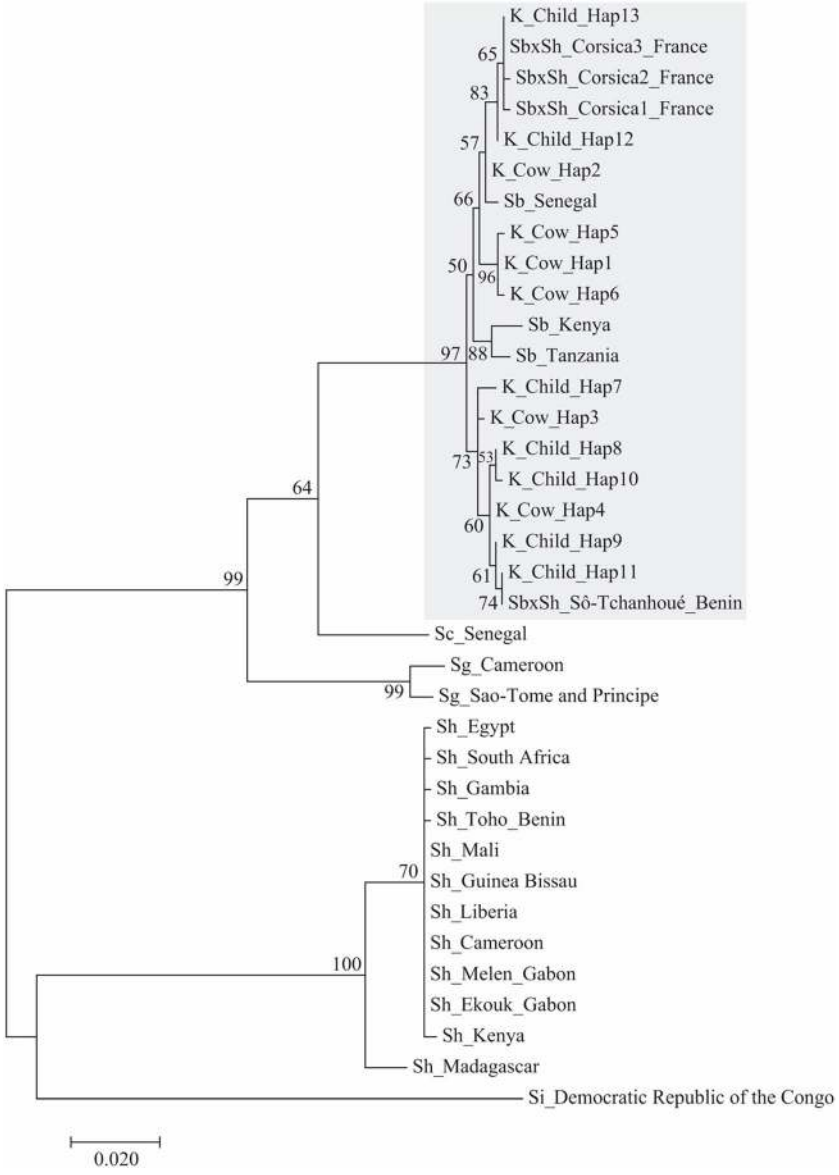


**Fig. 4** Maximum likelihood tree topology built with the 13 haplotypes COI mtDNA (881 bp) showing that *S. haematobium* from Kessounou (recovered either from cows or schoolchildren) belongs to the *S. bovis* clade. Haplotype code/COI mtDNA accession No: K_Cow_Hap1/MT159589, K_Cow_Hap2/MT159590, K_Cow_Hap3/MT159591, K_Cow_Hap4/MT159592, K_Cow_Hap5/MT159593, K_Cow_Hap6/MT159594, K_Child_Hap7/MT159595, K_Child_Hap8/MT159596, K_Child_Hap9/MT159597, K_Child_Hap10/MT159598, K_Child_Hap11/MT159599, K_Child_Hap12/MT159600, K_Child_Hap13/MT159601). The scale shows the number of nucleotide substitutions per site

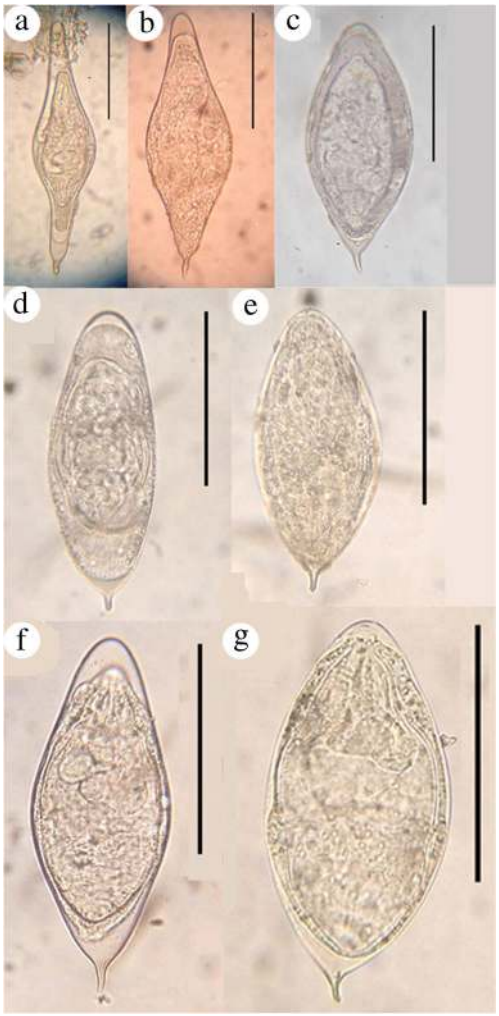


**Fig. 5** Egg morphology. Cow feces, **a** *S. bovis* morphotype, **b** intermediate morphotype, **c** *S. haematobium* morphotype. Schoolchild stool, **d** intermediate morphotype, **e** *S. haematobium* morphotype. Schoolchild urine, **f** intermediate morphotype, **g** *S. haematobium* morphotype. Bar represents 100 μm

The morphometry of the eggs from cows and schoolchildren is presented in Table 4 and Fig. 7. For the *S. bovis* morphotype, no comparison could be made since this morphotype was not present in schoolchildren. For the intermediate morphotype, mean egg length and spine length were significantly higher for the cow feces origin ($p < 0.05$) and no difference was observed between the urine and the stool of the schoolchildren ($p > 0.05$). The mean length/width ratio was significantly smaller for the eggs obtained from schoolchild urine ($p < 0.05$) and no differences were observed between the schoolchild and cow feces ($p > 0.05$). For the *S. haematobium* morphotype, no major difference was observed between the origins ($p > 0.05$). The comparisons between morphotypes indicated that mean egg length and egg length/width ratio from urine or from stool were significantly higher in *S. bovis* morphotype compared to the intermediate one and higher in the intermediate morphotype compared to the *S. haematobium* one ($p < 0.05$). The plot of the egg width (μm) and egg length (μm) for schoolchild urine (green diamonds), schoolchild stool (blue squares), and cow feces (orange triangles) showed that the eggs from schoolchild urine and stool were grouped together but that those of cow feces constituted a separate group, with some individuals making the link between the two groups showing a higher variability in the eggs from cows compared to the eggs from schoolchildren.

## Discussion

The results showed a chronobiological polymorphism in the cercarial emergence rhythm from snails exposed to miracidia collected from cow feces, with four different patterns.

The first pattern, early diurnal, exhibited a typical *S. bovis* cercarial emission; it was obtained in 7 *B. forskalii* among 11 (Kf1, Kf2, Kf3, Kf6, Kf7, Kf8, and Kf11). Such a pattern was found in *S. bovis* from Sardinia (Italy), Sudan and Spain

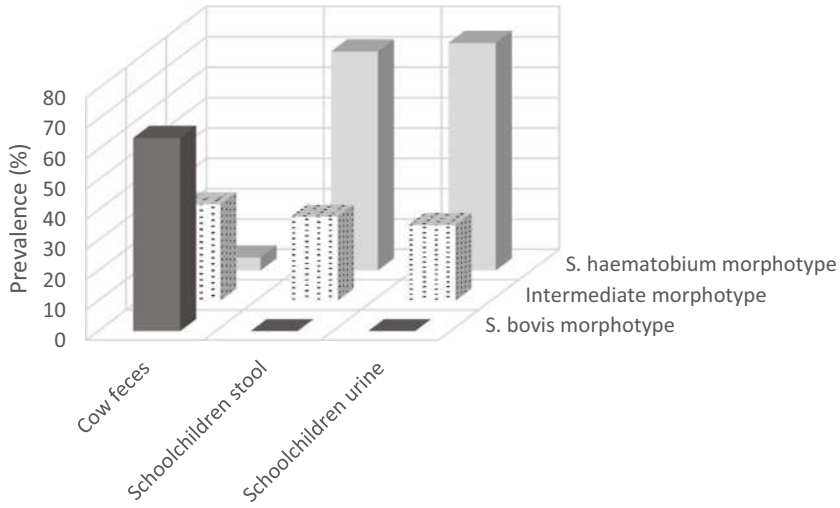


**Fig. 6** Percentages of each egg morphotype (*S. bovis*, black; intermediate, dots; *S. haematobium*, grey) according to origin (cow feces, schoolchild stool, schoolchild urine)

**Table 4** Egg morphometry (mean (μm) ± standard error)

| Morphotype | *S. bovis* | | | Intermediate | | | *S. haematobium* | | |
|---|---|---|---|---|---|---|---|---|---|
| Origin | CF | SS | SU | CF | SS | SU | CF | SS | SU |
| Length | 247.83[b] | na | na | 223.17[a, b] | 153.00 | 148.54[b] | 140.00 | 138.85 | 132.01 |
| SE | 4.03 | na | na | 7.51 | 5.48 | 3.82 | 42.43 | 4.68 | 1.66 |
| N | 30.00 | na | na | 15.00 | 5.00 | 12.00 | 2.00 | 13.00 | 36.00 |
| Min | 205.00 | na | na | 170.00 | 140.00 | 135.00 | 110.00 | 120.00 | 117.50 |
| Max | 300.00 | na | na | 262.50 | 170.00 | 170.00 | 170.00 | 175.00 | 170.00 |
| Width | 69.58[b] int | na | na | 83.17[a] | 56.00[a, b] | 68.13[a] | 67.50 | 68.85 | 68.96 |
| SE | 1.56 | na | na | 2.19 | 3.26 | 2.04 | 17.68 | 3.25 | 1.62 |
| N | 30.00 | na | na | 15.00 | 5.00 | 12.00 | 2.00 | 13.00 | 36.00 |
| Min | 57.50 | na | na | 65.00 | 50.00 | 60.00 | 55.00 | 50.00 | 50.00 |
| Max | 90.00 | na | na | 100.00 | 65.00 | 80.00 | 80.00 | 95.00 | 85.00 |
| Spine length | 17.38 | na | na | 17.31[a]SU | 13.33 | 10.94 | 17.50 | 10.31[a] | 12.59 |
| SE | 0.68 | na | na | 1.04 | 4.45 | 0.49 | 3.54 | 0.61 | 0.48 |
| N | 20.00 | na | na | 13.00 | 3.00 | 8.00 | 2.00 | 8.00 | 29.00 |
| Min | 12.50 | na | na | 10.00 | 7.50 | 10.00 | 15.00 | 7.50 | 10.00 |
| Max | 22.50 | na | na | 22.50 | 20.00 | 12.50 | 20.00 | 12.50 | 20.00 |
| Length/Width | 3.59[b] | na | na | 2.68[b] | 2.75[b] | 2.19[a, b] | 2.06[b] | 2.04[b] | 1.94 |
| SE | 0.07 | na | na | 0.05 | 0.11 | 0.07 | 0.09 | 0.07 | 0.04 |
| N | 30.00 | na | na | 15.00 | 5.00 | 12.00 | 2.00 | 13.00 | 36.00 |
| Min | 2.93 | na | na | 2.31 | 2.50 | 1.93 | 2.00 | 1.71 | 1.63 |
| Max | 4.17 | na | na | 2.93 | 3.10 | 2.72 | 2.13 | 2.50 | 2.60 |

*CF* cow feces, *SS* schoolchild stool, *SU* schoolchild urine, *SE* standard error, *N* number of eggs

[a] Mann-Whitney test: significantly different from the other origins of the same morphotype

[b] Mann-Whitney test: significantly different from the other morphotypes of the same origin

(Mouahid et al. 1987, 1991), and Niger (Mouchet et al. 1992). The COI mtDNA molecular analyses, conducted on cercariae from 2 snails among the 7 (Kf6 and Kf8), showed that the DNA sequences (K_Cow_Hap2) belonged to the *S. bovis* clade. *Schistosoma bovis* has an extensive geographical distribution (Moné et al. 1999), and in their review, Moné et al. showed that this species was naturally found in all of the neighboring countries of Benin: Togo, Burkina Faso, Niger, and Nigeria. However, while this is the first time that this species has been found naturally in Benin, this result is not surprising. Furthermore, if the presence of *S. bovis* was not surprising in cows, the presence of COI mtDNA *S. bovis* haplotypes in all of the schoolchildren samples confirms that introgressive hybridization between *S. bovis* and *S. haematobium* exists in humans in

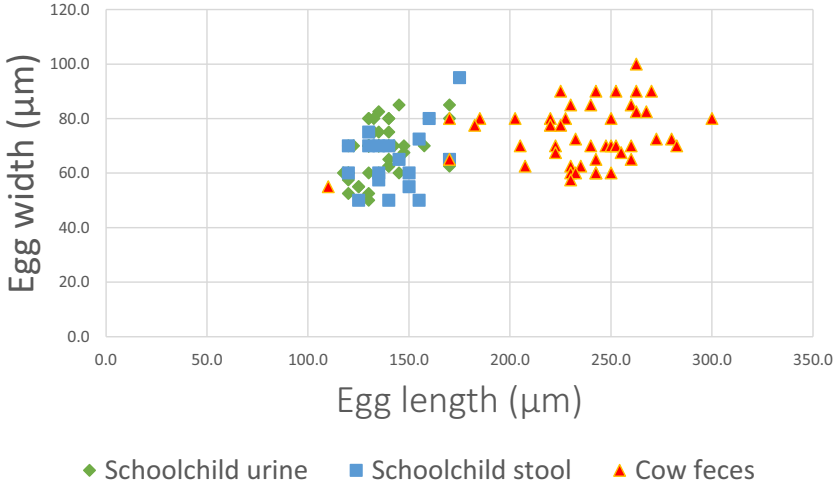


**Fig. 7** Plot of the egg width (μm) and egg length (μm) for schoolchild urine (diamonds), schoolchild stool (squares) and cow feces (triangles)

Benin, as shown previously by Moné et al. (2015). The phylogenetic analysis showed that 2 of the 6 haplotypes recovered from cows (K_Cow_Hap3 and K_Cow_Hap4) and 5 of the 7 haplotypes recovered from schoolchildren (K_Child_Hap7, K_Child_Hap8, K_Child_Hap9, K_Child_Hap10, and K_Child_Hap11) grouped with the one from Sô-Tchanhoué from Benin, confirms previous results on introgressive hybridization between *S. bovis* and *S. haematobium* with respect to humans in this area (Moné et al. 2015). The four last haplotypes recovered from cows (K_Cow_Hap1, K_Cow_Hap2, K_Cow_Hap5, and K_Cow_Hap6) grouped with the *S. bovis* haplotypes. The last two haplotypes recovered in humans (K_Child_Hap12 and K_Child_Hap13) grouped with those from Corsica, with the haplotype number 13 being exactly the same as the Corsica 3 haplotype. This result provides insight into the origin of the Corsican hybrids which was previously attributed to Senegal (Boissier et al. 2016) on the basis of 407 positions. The phylogenetic tree in the present work, based on 881 positions of the COI mtDNA, showed that the origin of Corsican haplotypes could be attributed to Benin.

The second pattern, midday to late diurnal, was new for cercarial emission in cows while it was similar to what has been published on *S. haematobium* in humans from Algeria (Kéchemir and Théron 1997), Morocco (Mouahid et al. 1991), Niger (Pagès and Théron 1990; Mouchet et al. 1992), and Gabon (Mintsa-Nguema et al. 2014). This is the first time that a typical *S. haematobium* pattern has been observed in cows as *S. haematobium* has always been thought to have the highest specificity to humans. This atypical pattern from cows was not due to the fact that the cercariae were shed by *B. globosus* (Kg2) since it has been shown that the taxa of the snail from which the cercariae emerge does not determine the cercarial emergence pattern of *S. bovis* (Mouahid and Théron 1986). Furthermore, hybridization in schistosomes is known to be accompanied by heterosis (hybrid vigor), including the capability of hybrid schistosomes to open their snail intermediate host spectrum (Wright and Ross 1980; Webster and Southgate 2003; Huyse et al. 2009). *Bulinus forskalii* is the main snail host for *S. bovis* in Sub-Saharian Africa while *B. globosus* is the main snail host for *S. haematobium* in this area (Brown 1994). The fact that a *B. globosus* was shedding this atypical pattern could stem from the opening of the snail host spectrum by this *S. bovis x S. haematobium* new pattern. The molecular and phylogenetic results presented in this paper support this idea since this *S. bovis* COI mtDNA haplotype (K_Cow_Hap4) grouped with the one from Sô-Tchanhoué from Benin harboring a *S. haematobium* x *S. bovis* genetic pattern (Moné et al. 2015). The molecular analyses also showed an atypical ITS rDNA profile for this sample (profile 11), with a *S. haematobium* pattern for ITS1 and double peaks for both 5.8S and ITS2 (Table 3). These results show that in this area, introgressive hybridization exists between *S. bovis* and *S. haematobium* in cows as well. This is the first time that such an introgressive hybridization has been identified in domestic bovines. Previous results showed introgression between *S. bovis* and *S. haematobium* in humans (Brémond et al. 1993; Huyse et al. 2009; Webster et al. 2013; Moné et al. 2015; Leger et al. 2016; Soentjens et al. 2016; Angora et al. 2019) and rodents (Catalano et al. 2018).

The third pattern corresponded to a new pattern for *S. bovis*, with both a typical early pattern for *S. bovis* accompanied by a nocturnal emergence peak at 7 pm. This pattern was obtained from 4 of the 11 *B. forskalii* snails (Kf4, Kf5, Kf9, and Kf10). This is the first time that a double peak in cercarial emergence is shown for *S. bovis*. Another animal schistosome, *S. margrebowiei* Le Roux, 1933 also showed two emergence peaks per day, with the first peak occurring 1 h after the onset of daylight, and the second peak 1 h after the onset of darkness (Raymond and Probert 1991). This is also the first time that a nocturnal cercarial emergence is shown for *S. bovis*. In another species of schistosome, *S. mansoni*, atypical chronotypes were found with nocturnal shedding. A three-peak cercarial emergence was found in southern Benin in both natural and experimental infections, with one peak in the early morning, a second around midday, and a third one at the beginning of the night (Ibikounlé et al. 2012). Another chronotype that displayed a strictly nocturnal emergence was shown in Oman and was related to the presence of reservoir hosts (rodents) in the transmission sites (Mouahid et al. 2012). However, this particular *S. mansoni* nocturnal chronotype was purely nocturnal and was not accompanied by diurnal cercarial emergence from the same individual snail. Several hypotheses could be proposed for the two peaks in diurnal-nocturnal cercarial emergence. First, the peaks may coincide with the behavior of the definitive host where cattle would go twice a day to drink and *S. bovis* would thus be adapted to this behavior. This explanation could also apply in the case of *S. margrebowiei*. Second, the peaks may be due to the presence of nocturnal reservoir hosts for *S. bovis*, like rodents, with nocturnal watering behaviors. This explanation would apply in the case of *S. mansoni* from Oman (Mouahid et al. 2012). Lastly, the peaks may have resulted in response to the presence of introgressive hybridization between *S. bovis* and a nocturnal population of *S. bovis* or another species of schistosome. It is difficult, however, to get a nocturnal pattern with two diurnal species of schistosomes (in our case, *S. bovis* and *S. haematobium*), even if they can hybridize. Pagès and Théron (1990) conducted crossing experiments between *S. bovis* from Spain and *S. haematobium* from Niger and the F1 hybrids harbored the same chronobiological pattern as the parental *S. bovis*, i.e., with an early peak. Unfortunately, these authors did not analyze the F2 generations. Furthermore, double infections were experimentally done with both *S. bovis* from Sardinia (Italy) and *S. haematobium* from Morocco and both species kept their own cercarial shedding rhythm in the doubly infected snails (Mouahid et al. 1991). This was also

the case for *S. mansoni* with both early and late diurnal patterns (Théron et al. 1997). The cercariae from 2 of the 4 snails (Kf5 and Kf9) used for cercarial emergence pattern were analyzed for molecular biology and showed *S. bovis* COI mtDNA haplotypes (K_Cow_Hap1 and K_Cow_Hap2). Both COI mtDNA haplotypes were found from different cercariae found in a single snail sample suggesting that, perhaps, 2 miracidia succeeded in developing in this one snail, leading to the double peak in cercarial emergence. However, as the other snail sample had only 1 COI mtDNA haplotype (K_Cow_Hap2) and yielded the same double peak pattern, this hypothesis is unsupported. The phylogenetic analysis showed that these COI mtDNA haplotypes (K_Cow_Hap1 and K_Cow_Hap2) grouped with the *S. bovis* haplotypes from other countries. The molecular analyses also showed typical ITS rDNA *S. bovis* profiles for these samples (profile 1).

The fourth chronotype, obtained from one *B. globosus* snail (Kg1), corresponded to a new chronotype for *S. bovis*, with both a typical *S. haematobium* late diurnal pattern accompanied by a nocturnal emergence peak at 7 pm. This is the first time that a midday to late diurnal peak together with a nocturnal peak was identified in *S. bovis*. This result shares similarities with the pattern with the previous one, with the presence of nocturnal cercarial shedding, but is different in that the early pattern in *S. bovis* was replaced by a late *S. haematobium* pattern. The molecular studies showed that this sample had the same COI mtDNA haplotype (K_Cow_Hap4) as the one linked to the second cercarial emergence pattern (*S. haematobium* midday to late diurnal pattern) and the phylogenetic analyses grouped this haplotype with the one from Sô-Tchanhoué, Benin. Furthermore, the ITS rDNA profile of all the cercariae in this sample (profile 10) showed a typical *S. haematobium* ITS1 marker together with a typical *S. bovis* ITS2 marker. Further studies on the behavior of the cattle and the presence of rodents as reservoir hosts are needed to explain this atypical pattern of cercarial emergence.

The results showed a polymorphism in egg morphology in both cows and schoolchildren, with 3 morphotypes. The first, the *S. bovis* morphotype, represented over 60% of the eggs in cow feces and no *S. bovis* morphotype was found in either the stool or urine of schoolchildren. Still, this result shows that 40% of the eggs recovered in the cows did not have a *S. bovis* morphotype and leads us to also suspect hybridization in cows between *S. bovis* and *S. haematobium*. With respect to egg morphometry, mean egg length of our *S. bovis* morphotype (247 μm) was similar to those recorded for *S. bovis* from cow feces (from 179 to 260 μm (Alves (1949); Pitchford (1965)). The second morphotype, *S. haematobium*, represented around 4% of the eggs in cow feces, compared to over 70% of the eggs in both stool and urine of the schoolchildren. This result showed that some eggs which harbored the *S. haematobium* morphotype were present in cows and that 30% of the eggs recovered in the schoolchildren lacked a *S. haematobium* morphotype, leading us to also suspect hybridization between *S. bovis* and *S. haematobium* in both cows and humans. Mean egg lengths of our *S. haematobium* morphotypes (132 to 140 μm) were similar to those recorded for *S. haematobium* eggs (from 131 to 146 μm; see Pitchford (1965), Loker (1983), Richard-Lenoble et al. (1993), and Moné et al. (2012)). The third chronotype, an intermediate morphotype between *S. bovis* and *S. haematobium*, sustained the hybridization between *S. bovis* and *S. haematobium*, as we would have suspected from the cercarial emergence results. Even if the shape of the eggs produced is not necessarily a guide to the genetic constitution of the enclosed larvae, as stated by Wright and Ross (1980), these authors also suspected natural hybrids between *S. haematobium* and *S. mattheei* thanks to *S. mattheei*-like eggs in a human infection. Mean egg lengths for our intermediate morphotypes, between *S. bovis* and *S. haematobium* (148 to 223 μm), also suggest the presence of hybridization between these two species in both cows and humans. However, much smaller intermediate egg sizes were found in the hybrids we found between *S. bovis* and *S. haematobium* in Corsica, France (Moné et al. 2015). A higher variability in the morphometry of the eggs was observed in cow feces compared to schoolchild stool or urine and thus place the cow host as the host which provides the largest panel of eggs for schistosome transmission in the area.

In conclusion, our results showed, for the first time, (i) the presence of *S. bovis* in Benin, (ii) the presence of introgressive hybridization between *S. bovis* and *S. haematobium* in domestic cattle, and (iii) the presence of atypical chronobiological patterns in schistosomes from cattle, with typical *S. haematobium* shedding pattern, double-peak patterns and nocturnal patterns. They also confirmed that introgressive hybridization between *S. bovis* and *S. haematobium* exists in humans in Benin. Very little comparison is possible between the present findings and the literature, because until now, the researchers who worked on introgressive hybridization had never analyzed the cercarial emergence patterns from naturally-infected cows. Our results showed that the chronobiological life-history trait is useful for the detection of new hosts and also may reveal the possible presence of introgressive hybridization in schistosomes. They also showed that another life-history trait, the prepatent period, which is known to be short for *Schistosoma bovis* (21–22 days, Mouahid and Théron 1987) and long for *Schistosoma haematobium* (32 to 43 days, Ibikounlé et al. 2013) was intermediate for all our samples (26 to 32 days).

Our results, for the first time, place cattle as reservoir host for *S. haematobium* and *S. bovis x S. haematobium*. The proximity between humans and cattle in Kessounou, like in many villages in Africa, is favorable for the interactions between their schistosomes, *S. haematobium* for humans and *S. bovis* for cattle. The consequences of these results on the epidemiology of the disease, the transmission to humans, and the control of the disease

are very important. They show that the definitive host spectrum of *S. haematobium* may be opened to include cattle; further research should be done regarding the importance of rodents, especially in nocturnal schistosomiasis.

Our results are not congruent with two recently published papers which suggest that hybridization may occur quite infrequently between *S. bovis* and *S. haematobium*. Boon et al. (2019) found strong differentiation between *S. bovis* and *S. haematobium* in human populations of the Senegal River Basin, suggesting that there is minimal gene flow between them. However, in their paper, they found a miracidium harboring a double *S. bovis* and *S. haematobium* ITS rDNA profile from a neighborhood village which was genetically highly differentiated from the others. They also questioned about the previous findings by Huyse et al. (2009) and Webster et al. (2013) showing the existence of contemporary hybrid crosses between *S. bovis* and *S. haematobium* in humans in Senegal. Platt et al. (2019) studied the exomes of miracidia coming from humans in Niger and Zanzibar and their data did not reveal any evidence for contemporary hybridization. Instead, they showed ancient introgression of some *S. bovis* alleles into the genome of *S. haematobium* in Niger. We are aware that our data were genotyped with just two loci (COI mtDNA and ITS rDNA) and that they may provide little power to detect recent hybridization. However, unlike other recent results, our data showed that double *S. bovis* and *S. haematobium* ITS rDNA profiles were not rare in Kessounou village suggesting a recent hybridization, as the ITS rDNA marker can retain both parental copies for several generations before they are homogenized by concerted evolution (Sang et al. 1995). Our results show that, in some places in Benin, gene flow between *S. bovis* and *S. haematobium* is present.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Ethical permission (No119, 17/07/2019/CER-ISBA) was obtained from the Ethic Committee of the « Institut des Sciences Biomédicales Appliquées, Faculté de Médecine, Université d'Abomey-Calavi, 01BP526, Cotonou, Benin.

**Informed consent** The Director of Kessounou school obtained informed consent from the parents of all the schoolchildren who participated to the study.

**Data set availability** Sequence data were deposited in the NCBI GenBank database under the accession numbers MT159589 to MT159601 for COI mtDNA and MT158872 to MT158882 for ITS rDNA.

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