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HEALTH ASSESSMENT OF CAPTIVE AND FREE-LIVING EUROPEAN POND TURTLES (*EMYS ORBICULARIS*) IN SWITZERLAND

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Abstract: The highly endangered European pond turtle (*Emys orbicularis*) was reintroduced in Switzerland in 2010. Up until 2019, no routine medical examinations have been carried out prior to its release or during recapture events. The aim of this study was to assess the health status of captive and free-living *Emys orbicularis* populations in Switzerland, taking into account the most important and frequently occurring health threats to freshwater turtles. A total of 141 European pond turtles, including captive (n = 89) and free-living (n = 52) individuals, underwent clinical examination (n = 136), choanal and cloacal swab collection for microbiology investigation (n = 140), blood sampling (n = 121), fecal examination for parasitology (n = 92), radiography (n = 84), and ultrasound (n = 46). Microbiology investigation included conventional PCR for herpesvirus, ranavirus, and *Mycoplasma* spp. Blood was used for the establishment of reference values for hematocrit, leukocyte count, and differential blood count as well as for biochemistry parameters tested with the VetScan VS2. An emydid *Mycoplasma* was detected in 40% (n = 56/140; 95%CI: 31.82–48.61%) of the turtles, including one individual with upper respiratory signs. Four animals positive for *Mycoplasma* arrived dead or were euthanized during the study period. Their necropsies revealed no evidence of respiratory disease. No ranavirus or herpesvirus was detected in any of the tested turtles. Two presumptively fatal infections with spirorchiid trematodes were reported during the study period. Endoparasites were detected in only 7.94% of the samples examined. This study provides comprehensive data on the current health status of the largest sample size of captive and free-living populations of *Emys orbicularis* ever assessed to date and serves as a baseline for future research investigations and management recommendations in this species.

INTRODUCTION

The European pond turtle (*Emys orbicularis*) is critically endangered in Switzerland.[34] Population decline has occurred across Europe and is mainly attributed to habitat loss and landscape disruption. Other threats include predation, water pollution, and competition by *Trachemys* spp., including the introduction of exotic pathogens.[14,21] Many European countries are committed to the protection of this critical species, including Switzerland, where the first official reintroduction of *Emys orbicularis* took place in 2010. Since then, this species has been bred in captivity and regularly released into the wild.[15] In compliance with the International Union for Conservation of Nature (IUCN) guidelines, preliminary medical examinations are imperative for a sustainable reintroduction of healthy *Emys orbicularis,* and a risk assessment focusing on known pathogens and diseases should be conducted.[20] For this reason, a standardized examination protocol was established in this study.

Information on infectious diseases affecting European pond turtles is scarce, but according to current literature, three pathogens—herpesvirus, ranavirus, and *Mycoplasma* spp.—appear to be of most significant importance in chelonians, including Emydid turtles.[1,10,24,40,41]

All three pathogens can cause similar clinical signs in chelonians, including nasal discharge, conjunctivitis, and additional, more general signs, such as lethargy and anorexia.[11] Hall-

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marks of herpesvirus infection in tortoises are stomatitis and glossitis, which can also be observed with ranavirus infection. Herpesvirus-associated necrotizing hepatitis is common in freshwater turtles, whereas ranavirus infection can case multisystemic disease. In marine turtles, common clinical signs of herpesvirus infection include a variety of skin lesions ranging from necrotizing to proliferative (neoplastic).[11,42]

Herpesvirus infections have been associated with mortality in captive individuals of the *Emydidae* family worldwide.[9,22,23] Furthermore, a herpesvirus (*Emydoidea Herpesvirus* 1) was identified in apparently healthy free-living emydid populations.[27] Ranavirus infections in *Emydidae* have been reported in North America and Europe; in Switzerland, ranavirus infection has been confirmed only in a captive Hermann's tortoise (*Testudo hermanni*).[17,29] Ranavirus is an emerging pathogen in both free-living and captive amphibian and reptile populations, and recent publications[30,38,41] indicate that it poses a greater threat to reptiles than previously thought. *Mycoplasma* spp. infection has been reported[1,36,37] both in apparently healthy free-living emydid turtles and in emydid turtles with clinical disease. There are no records in the current literature reporting the occurrence of clinical disease unambiguously causally associated with herpesviruses, ranaviruses, or *Mycoplasma* spp. in *Emys orbicularis*.

Among relevant parasites, a severe outbreak of spirorchiidiasis has been recorded[19] in Spain associated with blood flukes belonging to the species *Spirorchis elegans*. Furthermore, *Spirhapalum polesianum*—another spirorchiid trematode—has been reported[33] in free-living *Emys orbicularis* in Romania. In recent years, infestation with spirorchiid trematodes was associated with mortalities in various breeding facilities of *Emys orbicularis* in Switzerland.[26]

The aims of this study were three fold: (1) to conduct a comprehensive health assessment, (2) to understand the clinical relevance of three major chelonian pathogens in European pond turtles, and (3) to compare the pathogen and health status of free-living and captive animals.

This study includes the results of this first comprehensive health assessment, providing clinical and parasitological findings and reference values for critical health parameters, along with a preliminary estimated prevalence for *Mycoplasma* spp., herpesvirus, and ranavirus infections.

MATERIALS AND METHODS

Study population

Between June and August of 2019, 141 European pond turtles (*Emys orbicularis*) were examined. The animals in the nature reserves were captured with a system of semi-submerged nets and fishing traps (Fig. 1), in accordance with the RE-04 method approved by the Federal Office for the Environment. A PET bottle was placed in each trap to prevent the system from sinking below the water surface and to allow the turtle to breathe at all times.[16] The nets were checked once daily. All captured turtles were examined directly on site and released immediately after sampling. The animals at the breeding facilities were caught by hand or with a net.

Clinical examination and sampling

All animals were manually restrained and examined according to a standardized examination protocol. Biometric data were collected by weighing the animals and recording the straight carapace length (SCL). The animals were separated into groups by sex (male, female, undetermined) and age classes. Sex was determined on the basis of tail length, distance of the cloaca to the plastron margin, plastron concavity, and color of the iris.[45] Differentiation between juvenile and adult individuals is difficult, and no literature is available specifically for *Emys orbicularis*. The authors grouped individuals of <12 cm SCL as juveniles, because below this size a clear sex determination based on the above-mentioned characteristics was often not possible, and no eggs or large follicles were detected by diagnostic imaging (radiographs or ultrasounds) in animals below this size. The clinical examination was performed by the first author.

Separate choanal and cloacal swabs were taken from each animal using sterile cotton-tipped applicators with wooden shafts (Henry Schein, Lyssach, 3421, Switzerland) and then placed (the cotton tip only) in a 2-ml flat-cap microcentrifuge tube (Starlab, Muri, 5630, Switzerland) with 360 µl of ATL buffer (DNeasy Blood & Tissue Kit, Qiagen, Hilden, 40742, Germany). The tubes were stored at room temperature until DNA extraction. DNA extraction was performed either a few days or up to 2 wk after swab collection. Blood was collected from the dorsal coccygeal vein (*V. coccygealis dorsalis*) from individuals weighing more than 100 g and immediately transferred to an 0.5-ml lithium (LI)-heparin tube (Sarstedt, Sevelen, 9475, Switzerland). The max-

Figure 1. Capture system for free-living *Emys orbicularis*: The animals in the nature reserves were captured using a system of semisubmerged nets and fishing traps. The net directs the turtles into the trap. A PET bottle was placed in each trap to prevent the system from sinking below the water surface and to allow the turtles to breathe at all times. The systems were checked once daily. Illustration adapted to Cadi.[7]

imum amount of blood collected from each animal did not exceed 0.4% of body weight. Two blood smears were prepared immediately with blood from the Li-Heparin tube, and the remaining blood was cooled at +4°C. Feces were collected either from spontaneous droppings or from cloacal flushing with sterile saline solution. One part of the fecal material was stored at +4°C until further analysis, another part of the material was fixed in 12 ml of sodium acetate–acetic acid–formalin (SAF) solution and stored at room temperature.

All procedures were approved by the Cantonal Committee for Animal Experimentation, in accordance with the Swiss Animal Welfare Legislation (license No. BE 31/19).

Molecular diagnostics

Total DNA was extracted from combined choanal-cloacal swabs using a DNeasy Blood and Tissue Kit (Qiagen). DNA extraction was completed following the manufacturer's instructions. Total DNA amount and purity were measured for each sample using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA 02451, USA), and extracted samples were stored at −20°C until use. Conventional qualitative PCR was performed for herpesvirus, ranavirus, and *Mycoplasma* spp. PCR protocols can be viewed in a supplemental file (Supplement 1, which can be downloaded in the online article).

A nested consensus PCR was used for Panherpes screening according to an established protocol.[43] A positive control (US98/1976)[35] and water as a negative control were included in every batch. For the one-step ranavirus PCR, an established protocol[29] was adopted. A positive control (FV3 genomic DNA) and sterile water as a negative control were included. For the detection of *Mycoplasma* spp., an established protocol[5] amplifying the partial sequence of the 16S rRNA gene region was carried out. Total DNA from a diagnostic sample containing *Mycoplasma agassizii* DNA as positive control (obtained from *Gopherus agassizii*; CITES permits 16US50818A.9 and CH-27) and sterile water as a negative control were included.

A representative subset (three positive samples from each breeding facility and natural reserve, if available, equaling 24 samples in total) were further characterized using an additional PCR protocol[39] for the amplification of the 23S and 16S rRNA intergenic spacer region to further discriminate among the strains detected.

The samples from this representative subset ($n = 24$), including both the 16S and the 23S and 16S

intergenic region, were submitted for direct Sanger sequencing (both strands) to Microsynth (Balgach, 9436, Switzerland) and compared to known homologous sequences in GenBank using BLAST (Bethesda, Maryland, 20894, USA). The obtained sequences were manually assembled, aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation, version 5, Hinxton, CB10 1SD, UK), and then fed into MEGA 7 (Molecular Evolutionary Genetics Analysis, version 7.0 for bigger data sets, State College, Pennsylvania, 16801, USA).[25] Maximum likelihood phylogenetic analysis was carried out using standard settings with 1,000 bootstrap replications. Additionally, to the sequences obtained from this study, the following sequences were also computed in the tree: Uncultured emydid *Mycoplasma* clones (uncultured *Mycoplasma* spp. clone DE28, SL13, NJ15, PA2, NJ40, 17-598, DE26, 559/2008, C3), *Mycoplasma agassizzi* sequences (strain PS6 and AG37), a *Mycoplasma testudineum* sequence (strain BH29), and a *Mycoplasma gallopavonis* sequence (strain NCTC10186), which was used as an outgroup.

Blood analysis

Blood collected in Li-heparin tubes was used for hematocrit, a total leukocyte count, differential blood count, and blood chemistry within 4 h after collection. For the hematocrit, sodium-heparinized microhematocrit tubes (Medical Solution, Wil, 9500, Switzerland) were filled with the heparinized blood, spun, and read following the manufacturer's protocol. Leukocyte counts were determined manually using the Neubauer improved bright-line counting chamber (Medical Solution). For this procedure, 10 µl of blood was mixed with 490 µl of Natt and Herrick solution (Clinical Laboratory, University of Zurich, Zurich, 8057, Switzerland) and mixed for 5 min on a tumble-mixer (Sarmix GM1, Sarstedt AG, Sevelen, 9475, Switzerland) before the counting chamber was filled and analyzed according to an established protocol.[7] The blood smears of each animal were air-dried and stained with a modified Wright stain using the Hema-Tek 2000 slide stainer (Bayer, Münchenbuchsee, 3053, Switzerland) for differential blood count and blood parasite screening. Blood smears were evaluated by the first author and an experienced technician; 200 cells were counted for each smear, and means were recorded. If means differed greatly between the two evaluators, the count was repeated. Blood chemistry was evaluated with a VetScan VS2 (Abaxis Europe, Griesheim, 64347, Germany) using the avian-reptilian rotor.

Parasitology

Fecal material was stored in the fridge at +4°C and examined either immediately or, at the latest, within 5 d from sampling. A triple parasite screening, including a fresh smear, an iodine smear, and use of the SAF method, was conducted.[44] Because of the lack of material, no flotation was performed. For the iodine smear, one drop of R2 AxonGram solution (Axonlab, Baden, 5405, Switzerland) was used. Parasites were observed under ×100 and ×400 magnification with a Leica DMLB microscope (Ryf, Grenchen, 2540, Switzerland).

Diagnostic imaging

Three standard projections, including dorsoventral (DV), craniocaudal (CrCd), and laterolateral views, were acquired with a portable generator (SP-VET- 4.0 Sedecal, Alget, 28110, Spain) and associated digital radiography cassette system. For the DV view, patients were placed in ventral recumbency without any restraining aid. For the CrCd view, patients were restrained by placing them on top of a plastic jug and then they were simply rotated by 90° in order to achieve the desired position, with the animal facing the x-ray beam.

For the ultrasonographic examinations, a portable unit (MyLabDeltaVet Easote, Trezzano sul Naviglio, 20090, Italy) with associated microconvex transducer (SC3123: 4–9 MHz) was used. In all turtles, the two cervicobrachial and the two prefemoral windows were scanned, starting at the left prefemoral window and proceeding in a clockwise direction.

Both radiographic and ultrasonographic studies were reviewed by the co-author (BA) using an open-source DICOM commercial viewing software (Horos version 3.3.6, Annapolis, Maryland, 21401, USA).

Statistical analysis

The required sample size for estimating the prevalence of diseases was calculated with the Epitools Online calculator (Ausvet, Lyon, 69001, France). Descriptive statistics were tabulated for all continuous variables. Normality was assessed using the Shapiro-Wilk test, skewness, Q-Q plots, and kurtosis. Normally distributed data were compared for sex, age group, *Mycoplasma* infection, and habitat using an independent samples *t*-test. When data were not normally distributed, a Kruskal-Wallis one-way ANOVA was used. Cor-

Table 1. Demographic data of captive and free-living European pond turtles (*Emys orbicularis*) (n = 141) from Switzerland.

Origin	Institution/natural reserve	Location/canton	Adult female (No.)	Adult male (No.)	Adult unknown (No.)[a]	Juvenile (No.)
Captive	Berne Animal Park	Bern/Bern	10	2	0	11
Captive	Papiliorama	Kerzers/Freiburg	5	3	0	8
Captive	Private breeder	Gempen/Solothurn	7	7	1	1
Captive	Private breeder	Kriessern/St. Gallen	5	2	0	5
Captive	Private breeder	Menziken/Aargau	2	4	0	16
Free-living	Moulin-de-Vert[b]	Cartigny/Geneva	12	2	0	1
Free-living	Laconnex[c]	Laconnex/Geneva	9	4	0	2
Free-living	Prés-de-Villette[d]	Jussy/Geneva	4	2	1	5
Free-living	La Vieille-Thielle[e]	Cressier/Neuchâtel	4	6	0	0
Total			58	32	2	49

[a] Unknown: Tail length, distance of the cloaca to the plastron margin and plastron concavity were contradictory and therefore, sex determination could not be conclusively assessed.
[b] Coordinates: 46°10′N, 6°01′E.
[c] Coordinates: 46°09′N, 6°01′E.
[d] Coordinates: 46°15′N, 6°17′E.
[e] Coordinates: 447°02′N, 7°02′E.

relations between categorical variables were assessed using a chi-square test. The Mantel-Haenszel test was used to assess differences in categorical variables between habitats. Descriptive statistics and univariate analysis were performed using NCSS software (Kaysville, Utah, 84037, USA). In all cases, $P < 0.05$ was considered significant.

Reference intervals (RI) for hematology and blood chemistry were established following the ASVPC reference interval guidelines[14] using the Reference Value Advisor (RefVal, Version 2.1, National Veterinary School of Toulouse, Toulouse, 31300, France).[15] Outlier values were identified using the Dixon or Tukey range tests and were removed if attributable to a determinable biological reason. Lower and upper limits of the 95% RI, and their respective 90% CI, were calculated. For sample sizes 40 through 120, data were evaluated for normality as described above and robust methods applied to untransformed or Box-Cox–transformed (when normality was not achieved) data. If these methods revealed unreliable results, nonparametric methods were used. For sample sizes of <40, descriptive statistics only are reported.

RESULTS

Clinical examination

Between June and August 2019, 141 *Emys orbicularis* were examined, including 89 (63%) captive and 52 (37%) free-living individuals. An overview of the study population is provided in Table 1. Ninety-two animals with an SCL > 12 cm were classified as adult; 49 individuals with an SCL < 12 cm were classified as juvenile. Sex determination could be reliably performed on the basis of morphologic features in 95 animals (59 females = 41.6% and 36 males = 25.5%). Sex determination could not be conclusively assessed in 46 (32.6%) animals. Five dead animals were sent to the Berne Animal Park during the study period and were necropsied. Three animals showed clinical signs (lethargy and anorexia [n = 2], keratitis [n = 1], dyspnea [n = 1], and palpebral edema [n = 3]) and were therefore classified as "diseased." One of them had to be euthanized, and one died after a few days of treatment. From the total of seven necropsies conducted, four were not conclusive, one juvenile turtle died as a result of predation, and two animals died of an infestation with spirorchiid trematodes.

None of the necropsies revealed evidence of upper respiratory disease. The remaining 133 (94.3%) animals showed no detectable clinical signs or signs of active shell disease, although many of them showed variably extensive chronic shell lesions or other minor physical disorders. All clinical findings are summarized in Table 2. Most frequently, shell dermatitis of variable severity (n = 34/141, 24.1%), soft skin lesions (n = 22/141, 15.6%), and shell algae overgrow (n = 18/141, 12.8%) were observed. Shell dermatitis affected both the carapace and plastron and was more superficial on the carapace (consistent with erosive shell dermatitis) and deeper and more

Table 2. Clinical findings of captive and free-living European pond turtles (*Emys orbicularis*) (n = 141) from Switzerland. Thirty-three animals were affected by more than one abnormality.

Finding	Captivity (No., n = 89)	Free-living (No., n = 52)	Total (No.)
Apathy and anorexia	2	0	2
Dyspnea	1	0	1
Keratitis	1	0	1
Palpebral edema	3	0	3
Miosis	1	0	1
Shell covered with algae	2	16	18
Keratin proliferation of the shell or pyramidalization	2	4	6
Shell dermatitis	14	20	34
Traumatic shell lesion	7	2	9
Shell anomaly	2	2	4
Soft skin lesion (erosions, ulcerations, fibrotic scar, proliferations)	17	5	22
Soft tissue swelling	1	1	2
Tail deformation or tip loss	8	0	8
Jaw deformation	1	0	1
Limb amputation	1	1	2
Missing or shortened claw	8	1	9
Phalanx hypoplasia	2	0	2
Iatrogenic marking of the carapace	20	32	52
Turtles without lesions	36	23	59

severe on the plastron (consistent with ulcerative shell dermatitis, with associated dermal bone necrosis). Shell dermatitis and shell algae overgrow were significantly more frequently observed in the free-living population ($P = 0.04$ and $P < 0.01$, respectively), whereas there was no significant difference in the occurrence of soft skin lesions between the captive and free-living populations.

Molecular diagnostics

A total of 140 animals were tested by PCR for herpesvirus, ranavirus, and *Mycoplasma* spp. All tested turtles were negative for herpesvirus and ranavirus. *Mycoplasma* spp. was detected in 56 of 140 animals (40%, 95% CI: 31.82–48.61%). Although not statistically significant, the captive population had a higher prevalence (39/88, 44.32%; 95% CI: 33.73–55.30%) than did the free-living population (17/52, 32.69%; 95% CI: 20.33–47.11%). No significant difference in *Mycoplasma* prevalence was found between males and females (chi-squared test, $P = 0.4897$) nor between juveniles and adults (chi-squared test, $P = 0.6627$). There were no significant differences in *Mycoplasma* prevalence between habitats for either sex or age groups (Mantel-Haenszel test, $P = 0.8371$ and $P = 0.8509$, respectively). All breeding and free-living groups, with the exception of one free-living location (Moulin-de-Vert), included *Mycoplasma*-positive animals. All 16S sequences were identical and showed 100% nucleotide identity with already-published uncultured *Mycoplasma* strains from different authors from North America and Hungary (e.g., uncultured *Mycoplasma* spp. clone DE28 16S-23S, 559/2008 and SL13 16S).[3,12,36] There were minor differences in the sequences of the interspacer region reflecting the topography of the phylogenetic three (Fig. 2) and highlighting a small cluster of free-living animals (MT367553, MT367554, and MT367555) from the natural reserve in Laconnex.

Blood analysis

Blood was collected from a total of 121 of 141 animals (85.8%). Animals below 100 g in weight and dead animals were not sampled. Hematocrit was determined in 115 of 141 turtles (82%). Leukocyte counts were carried out in 82 of 141 animals (58%), and 76 blood smears of good quality (54%) were used for differential blood count references. Blood chemistry analysis was performed in 18 to 41 of 141 animals. Hematology and blood chemistry reference intervals are summarized in Tables 3 and 4. No blood parasites could be detected in any blood smear.

When correlating blood parameters with *Mycoplasma* infection, the hematocrit was significantly higher in *Mycoplasma*-positive individuals (*t*-test, $P = 0.004$). The relative monocyte count was significantly lower in *Mycoplasma*-positive samples (*t*-test, $P = 0.012$), while the absolute monocyte count revealed no significant difference between *Mycoplasma*-negative and -positive animals. None of the remaining hematologic and blood chemistry parameters differed significantly between *Mycoplasma*-negative and -positive individuals (*t*-test, $P > 0.05$).

Parasitology

Feces or cloacal lavage of 92 of 141 (65.2%) turtles from 52 individual and 11 pooled samples (from a total of 40 turtles) were examined. A fresh smear was prepared from all of them. On 55 of 63 (87.3%) samples, an iodine smear was performed. Additionally, 33 of 63 (52.4%) samples were tested with the SAF method. Parasite stages belonging to nematodes (eggs resembling genus

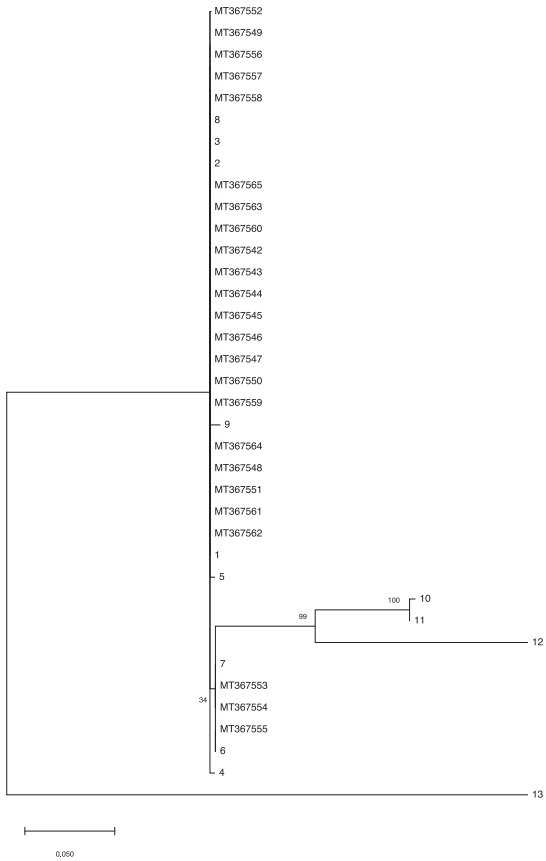

Figure 2. Phylogenetic tree based on the interspacer sequences obtained from 24 *Mycoplasma spp.* strains detected in European pond turtles (*Emys orbicularis*) (MT3675(xy)) and compared with the homologous sequences of uncultured emydid Mycoplasma clones (1–9),[a] *M. agassizii* sequences (10 and 11),[b] *M. testudineum* (12),[c] and *M. gallopavonis* serving as the outgroup (13)[d] using the Maximum Likelihood method and Tamura-Nei model. The tree represented here is the one obtained with the highest log likelihood (–3414.84). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. [a] 1= uncultured *Mycoplasma* sp. clone DE28 (GenBank Number [GN] KJ623616); 2 = uncultured *Mycoplasma* sp. clone SL13 (GN MG677114.1); 3 = uncultured *Mycoplasma* sp. clone NJ15 (GN KJ623620.1); 4 = uncultured *Mycoplasma* sp. clone PA2 (GN KJ623619.1); 5 = uncultured *Mycoplasma* sp. clone NJ40 (KJ623618.1); 6 = uncultured *Mycoplasma* sp. clone 17-598 (GN MH260079.1); 7 = uncultured *Falcaustra*; n = 2/63; 3.2%) and to protozoa (*Blastocystis* spp.; n = 1/63; 1.6%), trichomonads (n = 1/63; 1.6%), and hexamitids (n = 1/63 samples; 1.6%) were identified, as were eggs of monogenean (n = 1/63; 1.6%) and digenean (n = 2/63; 3.2%) trematodes. In Figure 3, selected images of specific parasites are shown. Pseudoparasites (*Malamoeba* spp., *Monocystis* spp., and *Ascaridia* spp.) were found in nine samples (n = 9/63; 14.3%). In seven samples, free-living ciliates and flagellates were present, a finding that is consistent with environmental contamination. In Figure 4, a selection of images from pseudoparasites found in this study is provided. Specific turtle parasites were found in 7.94% (5/63) of the native smears, 7.27% (4/55) of the iodine-stained smears, and 15.15% (5/33) of the samples tested with the SAF method. In three free-living and one captive individual (4/141 = 2.8%), parasitic trematodes were found in the oral cavity that were identified as *Polystomoides* spp. (Monogenea, Polystomatidae). Ectoparasites were not detected in any of the turtles.

In May and August 2019, two captive turtles were diagnosed with a fatal infestation with spirorchiid trematodes, presenting with severe chronic transmural enteritis, gastritis, hepatitis, nephritis with intralesional trematode eggs, and coelomitis.

Diagnostic imaging

Radiographs were taken of 84 (59.6%) randomly selected turtles, including 15 of 84 (17.9%) free-living and 69 of 84 (82.1%) captive individuals. Among these animals, 51 individuals (60.7%) were adult (31 females and 20 males), and 33 individuals (39.3%) were juveniles. Abnormal skeletal structures were observed in 19 of 84 (22.6%), showing mostly mild, multifocal radiolucent shell defects. Two free-living individuals from Moulin-de-Vert presented with extensive irregular multifocal to coalescing osteolytic regions of the carapace and plastron characterized by a moth-eaten pattern.

← *Mycoplasma* sp. clone DE26 (KJ623621.1); 8 = uncultured *Mycoplasma* sp. clone 559/2008 (GN FJ159565.1); and 9 = uncultured *Mycoplasma* sp. clone C3 (GN MH259305.1). [b] 10 = *Mycoplasma agassizii* strain PS6 (GN NR_025954.1); 11 = *Mycoplasma agassizii* isolate AG37 (GN MF966911.1). [c] 12 = *Mycoplasma testudineum* strain BH29 (GN AY366210.1). [d] 13 = *Mycoplasma gallopavonis* strain NCTC10186 (GN NZ_LR215031.1).

Table 3. Hematology reference intervals (RI) of European pond turtles (*Emys orbicularis*) without detectable clinical signs from Switzerland.

Parameter	n[a]	RI	Mean	Median	SD	90% LCI[b]	90% UCI[c]
Hematocrit (%)	115	3.6–24.6	14.3	14	5.3	2.5–5	23.1–26.1
Corrected leucocyte count (×10E3/µl)	82	0.57–8.47	3.37	3.3	1.72	0.28–0.83	6.11–9.07
Heterophils relative (%)	76	8.96–47.69	25.57	25	9.43	8.5–11.43	45.11–50
Eosinophils relative (%)	76	0.91–40.25	13.84	12	9.79	0.41–1.8	34.56–47.01
Basophiles relative (%)	76	4.19–33.8	18.99	19.25	7.37	2.16–6.48	31.34–36.29
Monocytes relative (%)	76	0.5–9.08	4.16	4	2.07	0.5–1.5	8.04–10
Lymphocytes relative (%)	76	12.89–68.41	37.19	36	13.25	11.5–19	63–73.5
Heterophils (×10³/µl)	76	0.13–2.19	0.86	0.78	0.53	0.09–0.21	1.88–2.55
Eosinophils (×10³/µl)	76	0.03–1.55	0.44	0.35	0.38	0.02–0.05	1.25–1.9
Basophiles (×10³/µl)	76	0.08–1.55	0.62	0.58	0.37	0.04–0.15	1.35–1.77
Monocytes (×10³/µl)	76	0.01–0.44	0.15	0.12	0.12	0.007–0.014	0.34–0.73
Lymphocytes (×10³/µl)	76	0.11–4.04	1.33	1.06	0.99	0.08–0.24	3.19–6.03

[a] n = 115 for hematocrit: includes 30 males, 51 females, and 34 individuals with undetermined sex (either juvenile or inconclusive sex determination); n = 82 for corrected leukocyte count: includes 24 males, 30 females, and 28 individuals with undetermined sex; n = 76 for the remaining parameters: includes 24 males, 27 females, and 25 individuals with undetermined sex.
[b] LCI = Lower CI.
[c] UCI = Upper CI.

Ultrasound was performed on 45 of 141 (31.9%) opportunistically collected large adults, including 14 of 45 (31.1%) free-living and 31 of 45 (68.9%) captive animals. Among these, 29 (64.4%) were females and 16 (35.6%) were males.

An individual with fatal spirorchiidiasis showed an increased amount of coelomic fluid between organs with slightly hyperechoic surfaces. Detailed diagnostic imaging results related to this case are currently underway.

DISCUSSION

With a sample size of 141 captive and free-living animals, this study allowed the authors to cover a representative portion of the Swiss *Emys orbicularis* population, estimated at 750 individuals (approximately 600 free-living and 150 breeding animals, according to S. Ursenbacher, pers. comm., 1 April 2019), providing a comprehensive and reliable assessment concerning their overall health parameters and a preliminary estimation of

Table 4. Blood chemistry reference intervals (RI) of European pond turtles (*Emys orbicularis*) without detectable clinical signs from Switzerland assessed with the VetScan VS2.

Parameter[a]	n[b]	RI	Mean	Median	SD	90% LCI[c]	90% UCI[d]
AST (U/L)	41	38.4–248	102.5	90	46.3	38–46.8	181.9–249
CK (U/L)	39	—	414.2	339	245	—	—
UA (µM/L)	31	—	42.5	34	38.3	—	—
Glu (mM/L)	41	1.9–9.81	4.74	4.6	1.96	1.63–2.33	8.31–11.44
tCa (mM/L)	40	2.09–4.23	2.73	2.58	0.5	2.03–2.18	3.53–5.69
Phos (mM/L)	41	0.53–2.19	0.95	0.86	0.31	0.53–0.59	1.47–2.22
TP (g/L)	41	15.2–72.2	33.2	32	11.2	15–20.2	56.3–73
Alb (g/L)	41	3–21	10	9	4.2	3–4.1	17.8–21
Glob (g/L)	18	—	25.6	20	12.6	—	—
K (mM/L)	40	2.9–7.88	5.42	5.15	1.18	2.43–3.37	7.14–8.36
Na (mM/L)	41	115.4–145	135.8	137	6.3	115–125	144.8–145

[a] AST = aspartate aminotransferase; CK = creatine kinase; UA = uric acid; Glu = glucose; tCa = total calcium; Phos = phosphate; TP = total protein; Alb = albumin; Glob = globulin; K = potassium; and Na = sodium.
[b] n = 40 for tCa and K: includes 14 males, 12 females, and 14 individuals with undetermined sex (either juvenile or inconclusive sex determination); n = 41 for AST, Glu, Phos, TP, and Na: includes 14 males, 13 females, and 14 individuals with undetermined sex; n = 39 for CK: includes 14 males, 11 females, and 14 individuals with undetermined sex; n = 31 for UA: includes 12 males, 10 females, and 9 individuals with undetermined sex; n = 18 for Glob: includes 6 males, 4 females, and 8 individuals with undetermined sex.
[c] LCI = Lower CI.
[d] UCI = Upper CI.

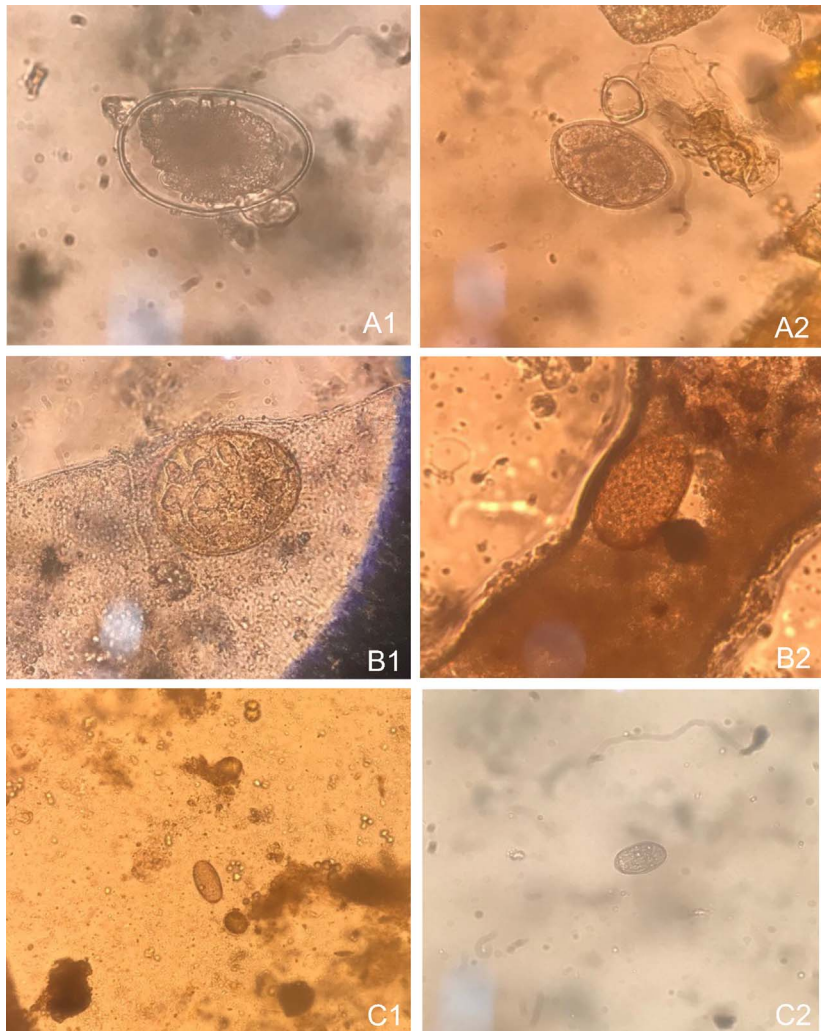

Figure 3. Selection of different parasites at distinct developmental stages in fecal samples of *Emys orbicularis* identified by direct smear (A2, B1, B2, C1) or SAF-technique (A1, C2). A1 and A2) Nematode eggs (*Falcaustra* spp.). B1 and B2) Monogenetic trematode eggs (polystomes). C1 and C2) Digenetic trematode eggs (telorchids).

Mycoplasma spp., herpesvirus, and ranavirus prevalence in this species. All animals were sampled during a single summer season to avoid the occurrence of potential seasonal bias on the assessed physiological parameters and pathogen detection.

Apart from four animals, all clinical findings were unremarkable. Other than shell lesions (keratin proliferation of the shell or pyramidalization, shell dermatitis, or traumatic shell lesion) in a total of 43 individuals, turtles appeared to be in good body condition, with adequate fat stores, and they showed no detectable clinical signs. Macroscopic shell lesions are frequent in free-living freshwater turtles, as described in a study on *Emys orbicularis* from Serbia,[2] with 59.33% of all animals diagnosed with shell lesions, with a variety of bacterial and fungal agents detected in shell swabs, or in a study from France,[4] with 21% of 3,636 *Emys orbicularis* presenting with shell lesions for which solitary causative agent could be identified. In the current study, histological examination of the upper keratin layer of some of the affected scutes revealed the presence of filamentous organisms, identified as common algae by PCR (data not shown). A follow-up study aiming to investigate the observed lesions by histopathology using full thickness shell biopsies has been planned.

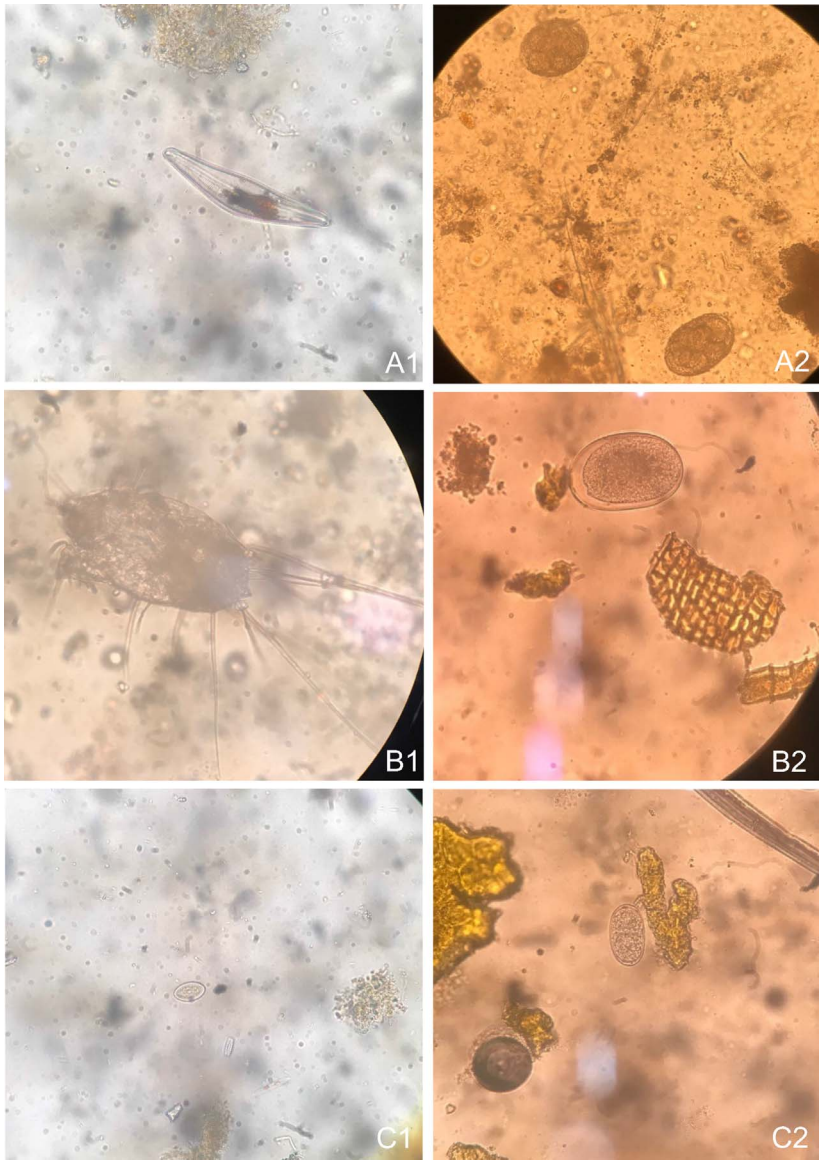

Figure 4. Selection of different pseudoparasites in fecal samples of *Emys orbicularis* identified by direct smear (A1, A2, B2, C1, C2) or SAF-technique (B1). A1) Plant structure; A2) Ascarid eggs (likely bird origin); B1) Free-living mite; B2) Egg of a free-living mite; C1) *Malamoeba* cyst; and C2) egg of a free-living nematode.

An emydid *Mycoplasma* was detected in 40% of all turtles, with a slightly higher, but not statistically significant, prevalence of 44.32% in captive animals. The higher values of captive animals may be explained by the higher population density per pond, which increases the risk of transmission. In the Northeastern United States, a high prevalence (58%) of emydid *Mycoplasma* in free-ranging emydid turtles was reported[36] in the absence of apparent clinical disease. Interestingly, most of the *Mycoplasma* sequences obtained in the current study share high similarities with uncultured clones from the United States (Fig. 2, Nos. 1–5). It can therefore be assumed that the American and European emydid *Mycoplasma* is likely either a single species or two distinct ones, but very similar ones, likely commonly present in freshwater turtles across the globe. The phylogenetic separation between the turtles from Laconnex (MT367553, MT367554, and MT367555) and those from the other natural reserves, although not statistically robust, fits with the most likely

origin of this group of turtles. Interestingly, these turtles were released in the 1950s through 1980s and did not originate from any of the breeding facilities that participated in the current study, possibly explaining the presence of a different variant of emydid *Mycoplasma*. Most likely, the *Mycoplasma* detected in this study was introduced into the environment, likely together with the originally introduced turtles, at least twice, in the 1950s and more recently in 2010. The increased hematocrit in *Mycoplasma*-positive animals could not be conclusively explained by the authors and might reflect sampling variations secondary to lymph contamination at the bleeding site. The clinical relevance of the lower relative monocyte count in *Mycoplasma*-positive animals is not clear and warrants further investigation.

Dyspnea was observed only in a single turtle out of those positive for *Mycoplasma*. *Mycoplasma* spp. are well-known pathogens in chelonians. Importantly, their significance as a primary pathogen has been conclusively demonstrated in tortoises, whereas the pathogenic potential and disease ecology of *Mycoplasma* spp. in freshwater turtles remain to be thoroughly explored. Interestingly, a very similar *Mycoplasma* to that detected in this study in European pond turtles was found in free-living freshwater turtles without apparent clinical disease in the United States.[36] However, in the absence of a transmission study to clarify whether the detected *Mycoplasma* could cause disease in naïve turtles, the principle of precaution should be applied when releasing animals that have tested positive for *Mycoplasma*. Accordingly, juvenile *Mycoplasma*-positive turtles were released only in ponds where the recipient turtle population already included *Mycoplasma*-positive individuals. Finally, a decision was taken to maintain the *Mycoplasma*-negative turtles in captivity for another season and to release them later on in a new location where no other turtles have ever been present. This setting provides the opportunity to set up a long-term investigation, aimed at better understanding the clinical significance and putative impact of *Mycoplasma* on *Emys orbicularis* populations.

No ranavirus or herpesvirus could be detected in this study. However, continuous monitoring is planned for the future, which the authors aim to complement with serological tests, which are particularly helpful in detecting latent or subclinical infections. As a result of financial and time constraints, in this study the authors focused on the three significant pathogens: *Mycoplasma* spp., herpesvirus, and ranavirus. For further investigations, other pathogens that may be of relevance in emydid turtles, such as adenoviruses or picornaviruses, should be considered as well.[11,12]

A sample size, ranging from 76 to 115 sample animals without detectable clinical signs or active shell disease, was available for measurement of hematological parameter, and this sample included captive and free-living animals. Because of financial constraints, only 41 samples could be analyzed with the VetScan VS2 for the establishment of blood chemistry parameters, which nevertheless allowed the authors to achieve an acceptable threshold.[13] Presently, the few reference values available for European pond turtles were obtained from a small sample size.[31,32] The reference intervals obtained in this study are comparable with the published values, even though the ranges were relatively wide, possibly reflecting known significant individual physiological variations.[8,18,28]

The most frequent radiographic findings were skeletal changes affecting the carapace and plastron of the turtles, consistent with the occurrence of shell dermatitis of varying severity. Although it is recommended that researchers perform lateral and CrCd views with a horizontal beam in order to obtain more conclusive radiographic findings, the authors decided to perform all projections with a vertical beam to reduce handling time and stress in the examined animals. The ultrasonographic findings in the *Spirorchis* spp.–infected turtle were consistent with the diffuse severe coelomitis recorded at necropsy. However, ultrasound could not detect lesions associated with its migration and the intralesional trematode eggs. Therefore, ultrasound is not considered to be a suitable detection method for infections with *Spirorchis* spp.

In this study, two cases of presumptive fatal infestation with spirorchiid trematodes occurred. Further research is needed to identify involved species and respective intermediate hosts, effective treatment strategies, and pertinent intravitam diagnostic tools. Accordingly, none of the animals from this pond, nor any animals that were in contact with the deceased individuals, were released into the wild.

CONCLUSION

The present investigation is the very first broad health assessment carried out in *Emys orbicularis* intended for release in Switzerland, and it fills a major knowledge gap. The use of a standardized examination method provided extensive information on the health status of the Swiss *Emys*

orbicularis population. Preliminary estimated prevalence was obtained for herpesvirus, ranavirus, and *Mycoplasma* spp., providing an important baseline for all future investigations. The parallel analysis carried out on captive and free-living animals provides an initial answer to recurrent questions concerning putative differences in health-related issues and parameters of captive and free-living animals. The results of this study have conclusively demonstrated for the first time the frequent presence of *Mycoplasma* spp. within the Swiss *Emys orbicularis* population in the absence of detectable clinical signs in the large majority of individuals, suggesting the existence of a possible host-pathogen equilibrium (co-evolution). Future studies are warranted to explore the significance of this emydid *Mycoplasma* in *Emys orbicularis* and its role in the disease ecology of this species.

This protocol will be adopted and constantly updated in light of future results for the selection of suitable animals to be reintroduced in Switzerland in the future.

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