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HOST MICROBE INTERACTIONS

Effects of stress exposure in captivity on physiology and infection in avian hosts: no evidence of increased *Borrelia burgdorferi* s.l. infectivity to vector ticks

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Abstract

Exposure to environmental stressors, an increasingly recurring event in natural communities due to anthropogenic-induced environmental change, profoundly impacts disease emergence and spread. One mechanism through which this occurs is through stress-induced immunosuppression increasing disease susceptibility, prevalence, intensity and reactivation in hosts. We experimentally evaluated how exposure to stressors affected both the physiology of avian hosts and the prevalence of the zoonotic bacteria *Borrelia burgdorferi* sensu lato (s.l.), in two model species—the blackbird *Turdus merula* and the robin *Erithacus rubecula* captured in the wild, using xenodiagnoses and analysis of skin biopsies and blood. Although exposure to stressors in captivity induced physiological stress in birds (increased the number of circulating heterophils), there was no evidence of increased infectivity to xenodiagnostic ticks. However, *Borrelia* detection in the blood for both experimental groups of blackbirds was higher by the end of the captivity period. The infectivity and efficiency of transmission were higher for blackbirds than robins. When comparing different methodologies to determine infection status, xenodiagnosis was a more sensitive method than skin biopsies and blood samples, which could be attributed to mild levels of infection in these avian hosts and/or dynamics and timing of *Borrelia* infection relapses and redistribution in tissues.

Keywords Reservoir host · Immunosuppression · *Borrelia* · Birds · Xenodiagnosis · Stress

Introduction

Natural communities are frequently exposed to stressors, including anthropogenic-induced habitat change and degradation [1]. Exposure to these stressors, especially during prolonged periods, has been proven to (a) cause immunosuppression in several vertebrate species, including humans and laboratory mice; (b) increase susceptibility to infectious agents; (c) influence the severity of infectious diseases; (d) diminish the strength of immune responses to vaccines, (e) reactivate latent infections and (f) slow wound healing [1, 2]. Wild animals are often chronically exposed to stressors such as competition, predation and strenuous activities as well as others that may directly compromise immune capacity, such as diminished food availability and quality, and exposure to immuno-toxic agents [3]. Therefore, changes in habitat structure or quality, either natural or anthropogenic, can influence the spread of diseases which are major factors modulating wild hosts' population processes and affecting biodiversity [4]. While it is often recognised that anthropogenic-induced habitat degradation has negative effects on the health of wild populations through increased disease spread and incidence [4, 5], it is generally neglected how overall environmental stress can impact zoonotic disease spread and infection risk to humans [6].

Wild birds are important in the enzootic cycle of severalpathogens, including the bacterial complex *Borrelia*

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burgdorferi sensu lato (s.l.), the etiologic agent of Lyme borreliosis. They are important hosts for the vector tick, and some species are competent reservoir hosts for *Borrelia* genospecies, acting as a source of infection to vector ticks, including genospecies that are pathogenic for humans and domestic animals (e.g., *B. garinii*; [7]). Thrushes have a key role in the maintenance of *B. valaisiana*, *B. garinii* and *B. turdi* in Europe [8–12], and were proven reservoirs for these three genospecies in experimental transmission studies [13–16].

The efficiency in transmitting the bacterium to ticks may vary over time, with some hosts establishing chronic infections and showing prolonged host-to-tick transmission (e.g., *Apodemus* species [17, 18]), while in others, the infectivity is more transient (e.g., *Turdus migratorius*; [19]). Therefore, spirochete location in host tissues, the duration of spirochetemia in blood, and spirochete load determine the availability of spirochetes to ticks, affecting efficiency of transmission [19–21]. In avian reservoir hosts, it has been reported that during periods of physiological stress, immunosuppression caused by elevated corticosterone may reactivate latent *Borrelia* infections, which escape control by the host's immune system, increasing the infectivity to ticks [16], and allowing isolation from blood and skin aspirates. However, the dermatropism of *Borrelia* has been reported in several wild reservoir hosts, with spirochetes being more often detected in skin biopsies [20, 22, 23] than in blood (but see [24]).

Energetic trade-offs between coping with environmental stressors and immune function may lead to infection relapses, and it is necessary to evaluate whether the exposure of vertebrate hosts to stress may impact the maintenance of pathogens in circulation and disease risk. In this study, we evaluated how two avian species act as reservoirs for *Borrelia* during stressful periods of their life cycle. We did this by assessing infection relapses after exposure to a stress protocol in captivity in blackbirds *Turdus merula* and robins *Erithacus rubecula* captured in two *Borrelia*-enzootic areas. These are ubiquitous bird species, common in both rural and urban areas, and important tick hosts. We used these two model species because previous evidence [10, 25–27] suggests that they differ in their reservoir competence. Prevalence of *Borrelia* in ticks derived from thrushes in the wild varies between 33 and 92% [26, 27]; while for the robin, the prevalence is generally very low [25, 27–31]. This last bird species, formerly classified as a member of the Turdidae family, has behavioural similarities to thrushes, foraging in shrubs and low vegetation, and, therefore, presents high tick infestation rates [32] as *Turdus* spp. do. Nonetheless, in Switzerland, *Borrelia*-infected larvae were detected by PCR in wild robins with a prevalence of up to 17%, and two isolates were obtained from the blood of this bird species [28, 29].

Simultaneously to the evaluation of avian infectiousness to vector ticks (through xenodiagnoses), we monitored changes in host physiology, and diagnosed infection in biological samples easily collectable during fieldwork (blood and skin biopsies) to (a) assess if they accurately reflect the infectious status of wild birds, and (b) if there are differences in infection reactivation between different tissues (reflecting local or systemic infections). In this study, we aim to better understand stress induction and its effects on infection dynamics in natural avian hosts using the tick-vectored infectious disease agent *Borrelia burgdorferi* s.l. as a model. This study will contribute to a better understanding of Lyme borreliosis eco-epidemiology by assessing how exposure to stress contributes to the maintenance of the pathogen, through its effects on reservoir hosts' physiology, including interactions with their immune capacity, and consequent impact on bacterial load and efficiency of transmission to vectors.

Methods

Bird Capture and First Tissue Sampling and Xenodiagnosis

Robins (n = 16) and blackbirds (n = 10) were captured in two enzootic areas approximately 160 km apart: Tapada de Mafra, Mafra (38° 57′ N, 9° 18′ W), and Mata Nacional do Choupal, Coimbra (40° 13 N, 8° 27′ W), Portugal, with mist nets, between 3 November 2016 and 25 January 2017. This period matches the peak activity of *Ixodes* sp. and falls outside the bird breeding period. At capture, birds were individually ringed and, when present, attached ticks were removed.

At capture (day 0), we measured the birds' body mass with a Pesola. A blood sample was collected from the brachial vein into (a) heparinised capillary tubes to make a blood smear and assess haematocrit, and (b) into an EDTA-coated tube for molecular detection of *Borrelia*. Additionally, blood from blackbirds was collected into another EDTA-coated tube for molecular detection of haemosporidia—this was not implemented for robins to minimise any complications due to transport and introduction into captivity, because of their smaller body size. Samples were kept at −80 °C until DNA extraction.

Birds were then transported in cardboard boxes to the lab and maintained in individual cages with food and water ad libitum. Food included granulated pellets suitable for thrushes, mashed fruit mix and blowfly larvae. On day 1, a skin biopsy (< 2mm^2) was collected from the chin area using a fine-point curved tweezer and a fine-tip curved scissor. Additionally, between 30 and 50 larval *Borrelia*-free *I. ricinus* from a laboratory colony (IS Insect Services GmbH, Berlin) were placed on each bird with a fine pencil under the head feathers (xenodiagnosis 1). The birds were immobilised in cotton bags for 1.5 to 2 h, to allow tick attachment. The birds were then placed back in their individual cages, and from day 4 to day 6, engorged xenodiagnostic larvae were collected from the trays

beneath the birds (see [15] for further details) and preserved in 70% ethanol (see Fig. 1 for a detailed timeline of experimental procedures).

Maintenance in Captivity and Stress Protocol

Birds' body mass was measured at days 7, 15 and 22. At day 7, the birds were randomly divided into two groups: control versus exposure to chronic stress [33, 34] in a stratified sampling design by age (adult/first year) and sex (for blackbirds only). At this point, we had no information on the infection status of the birds. In the stressed group, the birds were exposed, over 9 consecutive days during daylight hours, to 4 stressors in random order: 60 min of loud music radio (over 80 dB), 60 min of restraint in a cloth bag, 30 min of chasing and cage tapping (for 15 s at 2-min intervals) and 30 min of crowding (maintaining 3 individuals in the same cage, for robins only) or rolling the birds' individual cages on a cart (for blackbirds only).

This stress protocol has been previously used in passerines to induce chronic stress in captivity and evaluate its impacts in hormonal responses, including corticosterone levels, plasma metabolites, immune response and senescence biomarkers [33–35]. These stressors significantly decreased the weight in European starlings (*Sturnus vulgaris*) and blackbirds after 5–10 days of exposure to the stress protocol [33, 35],increased stress-induced (to handling) corticosterone response while decreasing corticosterone levels after 9–11 days in European starlings [36], and increased oxidative damage 10 days after exposure in blackbirds [33]. Birds in the experimental group were moved out of the common room where the birds were held in their individual cages in order to apply the stressors. Control birds were always kept in their individual cages and their disturbance was kept to a minimum.

Final Tissue Sampling and Xenodiagnosis

On the last day of exposure to the stress protocol (day 15), a blood sample was collected into a heparinised capillary tube to make a blood smear, and to evaluate haemosporidian infection (in blackbirds only). At day 16, we collected a second skin biopsy and performed a second xenodiagnosis (xenodiagnosis 2) to assess *Borrelia* infection reactivation. After all xenodiagnostic ticks had dropped off, at day 22, we collected a blood sample into both heparinised capillary tubes and EDTA-coated tubes, to make a blood smear, evaluate *Borrelia* reactivation in blood and haemosporidian infection (in blackbirds only; see Fig. 1 for a detailed timeline of experimental procedures). We also collected an aspirate from a foot nodule developed by one of the robins during the captivity period. On day 23, the birds were released in the same locations they were captured.

Laboratory Analyses

The blood smears were air-dried, fixed with methanol and stained with Giemsa. The blood smears were observed using a microscope under oil immersion to estimate the number of white blood cells per 10,000 red blood cells (WBC). Heterophil to lymphocyte ratios (H/L) were assessed by identification of 100 white blood cells [37]. These immune indices were monitored to obtain information on the status of the

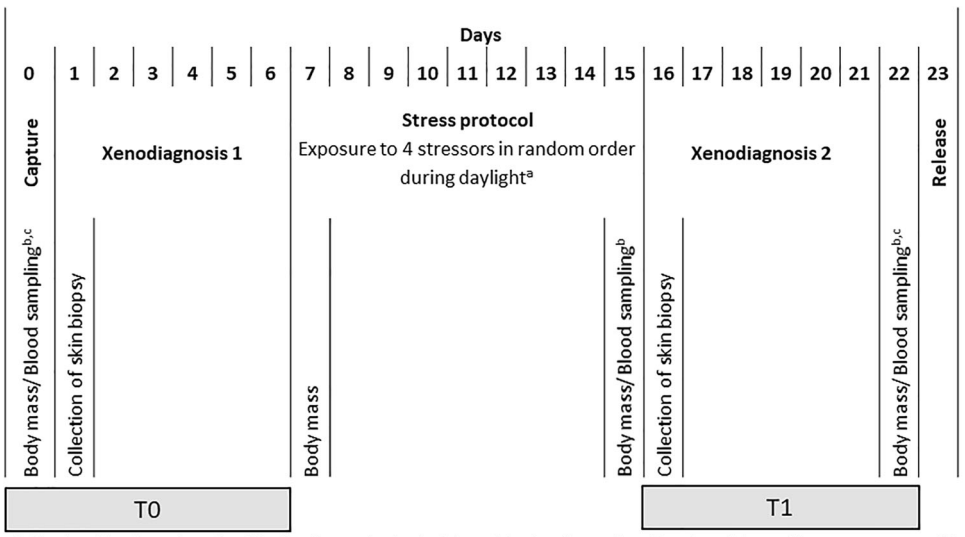

Fig. 1 Timeline of experimental procedures and sample collection from wild blackbirds *Turdus merula* and robins *Erithacus rubecula*

[a] 60 min of loud music radio, 60 min of restraint in cloth bag, 30 min of crowding (*E. rubecula*) or rolling cage on a cart (*T. merula*), and 30 min chasing/ cage taping (15s every 2m) - applied to the stress-exposed treatment group.
[b] Evaluation of number of heterophils, number of lymphocytes, WBC, heterophil/lymphocyte ratio, haematocrit and malaria (*T. merula* only).
[c] Evaluation of blood infection status by *B. burgdorferi* s.l.
T0 – first sampling; T1 – final sampling

immune system and because H/L ratio is a widely used generalist stress indicator in birds in ecophysiological studies [36, 38]. Haematocrit was measured after centrifuging the capillary tubes containing the blood samples at 14,000*g* for 10 min.

DNA was extracted from engorged ticks and bird tissues with DNeasy blood and tissue kit (Qiagen, Hilden, Germany). *Borrelia* infection was assessed through real-time PCR targeting the 23S rRNA [39]. A quantitative polymerase chain reaction (qPCR) with a serial dilution quantification standard of *B. turdi* in a LightCycler 480 (Roche Diagnostics, Pleasanton, CA, USA) was used to analyse *Borrelia* infection in ticks. DNA was extracted from the *B. turdi* isolate T2084A (3rd passage; [40] using the Qiagen DNAeasy blood and tissue kit). DNA was eluted in 100 μl of ultrapure DNAse-free water and its concentration estimated with Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration, assuming a genome size of 1.3 × 10^6 bps [41] for *B. burgdorferi* s.l., was used to calculate the number of genome copies. We then created a serial dilution of standards ranging from 10 to 10^6 genome copies/μl. We used the estimation of the number of *B. burgdorferi* s.l. genome copies in each tested sample given by the cycler standard curve to extrapolate the number of *B. burgdorferi* s.l. genome copies in each of the xenodiagnostic ticks. All quantitative PCR runs included seven negative controls throughout the plate and had efficiencies above 1.8. The *flaB* gene of positive samples was amplified in a nested PCR using the primers described in [42], and the amplicons were sequenced at the sequencing unit of the National Institute of Health Doutor Ricardo Jorge, to identify the *Borrelia* genospecies, after analysis using DNAstar 7.1 (Lasergene, Wisconsin, USA) and comparison with reference sequences using Blast analysis (http://blast.ncbi.nlm.nih.gov/).

Blackbirds and other passerines are known to often carry haemosporidian parasites [43] which could potentially affect their physiology. The presence of haemosporidian parasites (*Plasmodium* sp., *Haemoproteus* sp. and *Leucocytozoon* sp.) was detected by PCR amplification of a fragment of their mitochondrial DNA cytochrome-B gene in blackbirds. The protocol uses three pairs of primers, with the same forward (UNIVF) and three different reverse primers (UNIVR1, UNIVR2 or UNIVR3; [44, 45]). All PCRs consisted of 0.9 μl of HOT FIREPol Blend Master Mix 10.0 (Solis BioDyne, Estonia), 0.35 μl of each primer, and 1 μl of DNA template for a total volume of 8.1 μl. We used the following conditions: 15 min at 95 °C, 37 cycles of 30 s at 95 °C, 30 s at 51 °C and 90 s at 72 °C, followed by 10 min at 72 °C. All PCR sets were accompanied by negative and positive controls (from foreign lineages endemic to the Caucasus region) to detect PCR success and possible contaminations. The PCR result was visualised using a FluoroImager (Molecular Dynamics, USA) after DNA fragment separation by electrophoresis in a 2% agarose gel. We considered a bird to be infected with malaria if the PCR was positive for any of the primer sets.

Statistical Analyses

Variation in Avian Physiological Parameters

Variation in the physiological parameters (body mass, haematocrit, WBC, number of heterophils, number of lymphocytes and H/L ratio) of each species was evaluated using linear mixed effects models. In these models, the fixed effects structure included the categorical factors: day of experiment (3 levels for all parameters, except 4 levels for body mass), treatment (stress/control) and interaction between these terms. *Borrelia* infection status, study area (for robins) and presence of malaria parasites (for blackbirds) were also included in the models. Bird identity was included as a random effect. We ran a set of models with different fixed effects structures and used the Akaike Information Criterion (AIC; Online Resource 1) to select the best model which we used to calculate conditional model-averaged parameter estimates. Significance level was defined at $P = 0.05$. H/L ratio was log-transformed, and WBC counts were square root–transformed for normality. *Borrelia* infection status at each day of physiological evaluation was included by combining information available from blood, skin biopsies and xenodiagnostic ticks collected closer to each of the physiological sampling days: (a) for days 0 and 15, *Borrelia* infection included information from blood, skin biopsies and xenodiagnostic ticks for blackbirds, and information from skin biopsies and xenodiagnostic ticks for robins; for day 22, only information from blood was available for both bird species. The study area was only included when modelling robin's physiology because there were enough observations from each of the study areas to allow statistical analyses (Tapada de Mafra $n = 11$; Mata do Choupal $n = 5$), contrary to blackbirds (Tapada de Mafra $n = 2$; Mata do Choupal $n = 8$).

Variation in *Borrelia* and Haemosporidian Infection

The effects of stress on *Borrelia* infection relapses were evaluated in terms of prevalence and spirochete load in xenodiagnostic engorged larvae, and prevalence in blood and skin biopsy tissues. This was compared between stress and control groups, and between sampling periods—first sampling (T0: blood - day 0; skin biopsy - day 1; xenodiagnosis 1 - day 1 to 6)—and final sampling, i.e. after exposure to the stress protocol in captivity (T1: skin biopsy - day 16; xenodiagnosis 2 - days 16 to 21; blood - day 22). For the presence of *Borrelia* in the blood of blackbirds, a 3rd sampling between T0 and T1 was considered. This was evaluated by fitting a zero-inflated generalised linear mixed models (ZIGLMM) with binomial distribution (logit function) to model the presence of

Borrelia infection in the birds' blood, skin biopsies and xenodiagnostic ticks, and Poisson distribution (log function) to model *Borrelia* prevalence and spirochaete load in xenodignostic ticks. The models included treatment (stress/control), timing of experiment (T0/T1, except for presence of *Borrelia* in the blood of blackbirds where a 3rd sampling between T0 and T1 was included), bird species (blackbird/robin; except for presence of *Borrelia* in blood-modelled only for blackbirds), and study area (Mata do Choupal/ Tapada de Mafra; for robins only) as fixed effects. Individual ID was included as a random effect to control for different non-independent assessments of the same individual at different time points of the experiment.

Variation in the presence of malaria parasites in blackbirds' blood was assessed using zero-inflated generalised linear mixed models (ZIGLMM) with binomial distribution (logit function). Day of experiment (3 levels), treatment (stress/control) and interaction between these terms were included in the fixed effects structure, and bird identity was included as a random effect. Analyses were performed using the packages glmer/lme4, glmmTMB and MuMin in R [46].

We assessed if infection by malaria had any significant association with infection by *Borrelia* (either in blood or assessed by combining results from blood, skin biopsies and xenodiagnostic ticks) in blackbirds in each of the experimental periods using Fisher's exact tests.

Comparison of *Borrelia* Infection Diagnosis Techniques—Skin Biopsies, Blood and Xenodiagnoses

We compared prevalence of infection in birds obtained through the analysis of blood or skin biopsies, with that obtained by analysing xenodiagnostic larvae, within sampling periods (T0 and T1), using Fisher's exact tests. Comparison between blood analysis and xenodiagnosis at T0 was not statistically possible because there were no blood positive samples at this sampling period. Additionally, we calculated the sensitivity and specificity of blood and skin biopsy analysis using xenodiagnoses as the standard method following Feinstein [47] and tested the probability of agreement and symmetry between results of the different tests (xenodiagnosis versus blood samples and xenodiagnosis versus skin biopsies) using McNemar's test. Data are reported as mean ± SE, and the significance level was defined at $P < 0.05$.

Results

Variation in Avian Physiological Parameters

Body mass of robins and blackbirds were both significantly affected by the day of experiment (Table 1), meaning that both bird species lost weight during the experiment, especially from day 0 to day 7, after which birds slowly regained weight (Fig. 2a).

In robins, heterophils increased significantly during the course of the experiment, and this was more clear in the stressed group (Table 1; Fig. 2b) Consequently, day of experiment had a significant effect on robins' H/L ratio, which increased during the experiment (Table 1; Fig. 2c). In blackbirds, heterophils were affected by an interaction between day of experiment and treatment (Table 1; Fig. 2b): heterophils presented an overall increasing trend during the experiment but, in the stressed group, they decreased from day 15 to day 22 (after the experimental exposure to stressors ceased). This trend was also apparent for H/L in blackbirds but it was not significant (Table 1, Fig. 2c).

White blood cell counts were significantly affected only by the presence of malaria in the blood of blackbirds (Table 1; mean WBC in blackbirds with malaria infection ± SE = 28.1 ± 2.76, $n = 19$; non-infected blackbirds = 17.18 ± 2.64, $n = 11$). There were no effects of day of experiment, treatment, *Borrelia* infection, study area (for robins) and malaria infection (for blackbirds) on the number of circulating lymphocytes and haematocrit.

Variation in *Borrelia* Infection Levels

Borrelia Infection Levels—Effect of Bird Species

Overall *Borrelia* prevalence was 69.2 (18/26; infection prevalence in robins = 50%; blackbirds = 100%) when considering that a bird was infected when at least one of the tests (xenodiagnosis, analysis of skin biopsies or blood) was positive at any given time point of the experiment. A significantly higher number of blackbirds produced *Borrelia*-infected xenodiagnostic larvae comparatively to robins (total number (T0 + T1) of blackbirds with infected xenodiagnostic ticks = 15 out of 20; robins = 3 out of 32; estimate = 1.23 + 5.85, $Z_{1,50} = 2.07$, $P = 0.038$). *Borrelia* prevalence in xenodiagnostic larvae was also significantly affected by bird species (prevalence in xenodiagnostic larvae fed on blackbirds = 44.28% ± 9.2%, $n = 20$; robins = 1.34% ± 0.75%, $n = 32$; estimate = 32.0 + 9.93, $Z_{1,50} = 3.14$, $P = 0.0017$). Similarly, spirochete load in xenodiagnostic larvae was also significantly higher in xenodiagnostic ticks fed on blackbirds than on robins (mean number of spirochetes in xenodiagnostic ticks fed on blackbirds = 1811 ± 622.32, $n = 140$; robins = 0 ± 0.0, $n = 224$; estimate = 30.47 + 5.89, $Z_{1,363} = 5.16$, $P < 0.0001$).

Borrelia Infection Levels—Effect of Experimental Procedures and Stress Protocol

There was no significant effect of the stress protocol on *Borrelia* infection in xenodiagnostic ticks (both prevalence and spirochaete load; Table 2; Fig. 3). This

Table 1 Conditional model-averaged coefficient parameters from the generalised linear mixed models (GLMMs) that best explained (lowest AICc) variation in physiological parameters of robins *Erithacus rubecula* and blackbirds *Turdus merula*. Fixed effects included day of experiment (3 to 4 levels depending on parameter measured), treatment (stress or control), and interaction between these terms. The presence of *Borrelia* infection (infected/non- infected), presence of malaria infection (infected/non-infected; blackbirds only) and study area (Mata do Choupal/Tapada de Mafra; robins only) were also included as fixed effects. Individual ID was included as a random effect. WBC was square root–transformed, and H/L ratio and number of heterophils were log-transformed for normality. Only the models in which one of the fixed effects or their interactions was significant (in bold) are presented

	Bird species	*n*	Day of experiment			Treatment			Day of experiment * treatment			Malaria infection			*Borrelia* infection			Study area			Malaria infection * *Borrelia* infection		
			Estimate± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*	Estimate ± SE	*Z*	*P*
Body mass (g)	Robins	**63**	**−0.054 ± 0.014**	**3.9**	**< 0.001**										0.54 ± 0.35	1.47	0.14						
	Blackbirds	**39**	**−0.36 ± 0.074**	**4.61**	**< 0.001**							1.81 ± 1.58	1.08	0.28									
Heterophils	Robins	**47**	**0.854 ± 0.34**	**2.43**	**0.015**	−2.08 ± 9.40	0.22	0.83	0.84 ± 0.49	1.67	0.095							1.25 ± 8.28	0.15	0.88			
	Blackbirds	**30**	**1.12 ± 0.30**	**3.55**	**<0.001**	2.8 ± 7.3	0.37	0.71	**−0.98 ± 0.36**	**0.38**	**0.0095**	−8.06 ± 4.06	1.89	0.059	8.68 ± 4.38	1.87	0.06						
H/L ratio	Robins	**47**	**0.065 ± 0.016**	**9.94**	**<0.001**																		
WBC	Blackbirds	**30**										**2.17 ± 0.85**	**2.41**	**<0.001**	0.23 ± 0.70	0.31	0.76				−1.32 ± 1.01	1.23	0.22

result suggests that the stress protocol itself did not reactivate *Borrelia* infection. Day of experiment and study area also did not affect *Borrelia* infection in xenodiagnostic ticks. *Borrelia* prevalence in the blood of blackbirds increased along the experiment: *Borrelia* prevalence at capture was zero, whereas 60% of blackbirds showed infection in their blood at day 22 (Table 2; Fig. 3), suggesting reactivation of *Borrelia* infection during captivity. *Borrelia* infection in skin biopsies was not affected by either day of experiment, treatment, study area or bird species.

One robin from the control group, which had a positive skin biopsy at first sampling, but no other positive samples throughout the experiment, developed a swelling in the foot during the second xenodiagnosis. A smear from this nodule aspirate revealed a high number of heterophils in the fluid but was negative for *Borrelia* by real-time PCR.

Borrelia Genospecies in Avian Tissues and Xenodiagnostic Ticks

From the eight infectious blackbird individuals to xenodiagnostic ticks, five transmitted *B. turdi* to feeding xenodignostic larvae, and one transmitted *B. valaisiana* and *B. turdi*. It was not possible to identify the genospecies present in engorged positive ticks from the remaining two blackbirds, and the genospecies transmitted by the three infectious robins, possibly due to low levels of infection. *Borrelia turdi* was the genospecies present in one out of the five positive biopsies, which was collected from a blackbird that transmitted *B. turdi* to xenodiagnostic larvae. Positive blood samples were infected with *B. turdi* (4/11), including one blood sample collected from a robin.

Haemosporidian Infection and Interactions with Borrelia Infection

All individual blackbirds were positive for malaria infection at least for one of the three sampling time points (Online Resource 2). Two individuals tested positive in all the three sampling times. Three (3/10) blackbirds were negative at first sampling and tested positive in further samplings, whereas two individuals were positive for malaria in the first sampling only. The remainder tested positive at capture and at the end of the captive period (n = 3). There was a tendency, albeit not significant, for a higher probability of a blackbird to be infected with malaria parasites when infected with *Borrelia* at a given sampling point (assessed by combining the results from blood, skin biopsies and xenodiagnostic ticks) (Fishers' exact test P = 0.091).

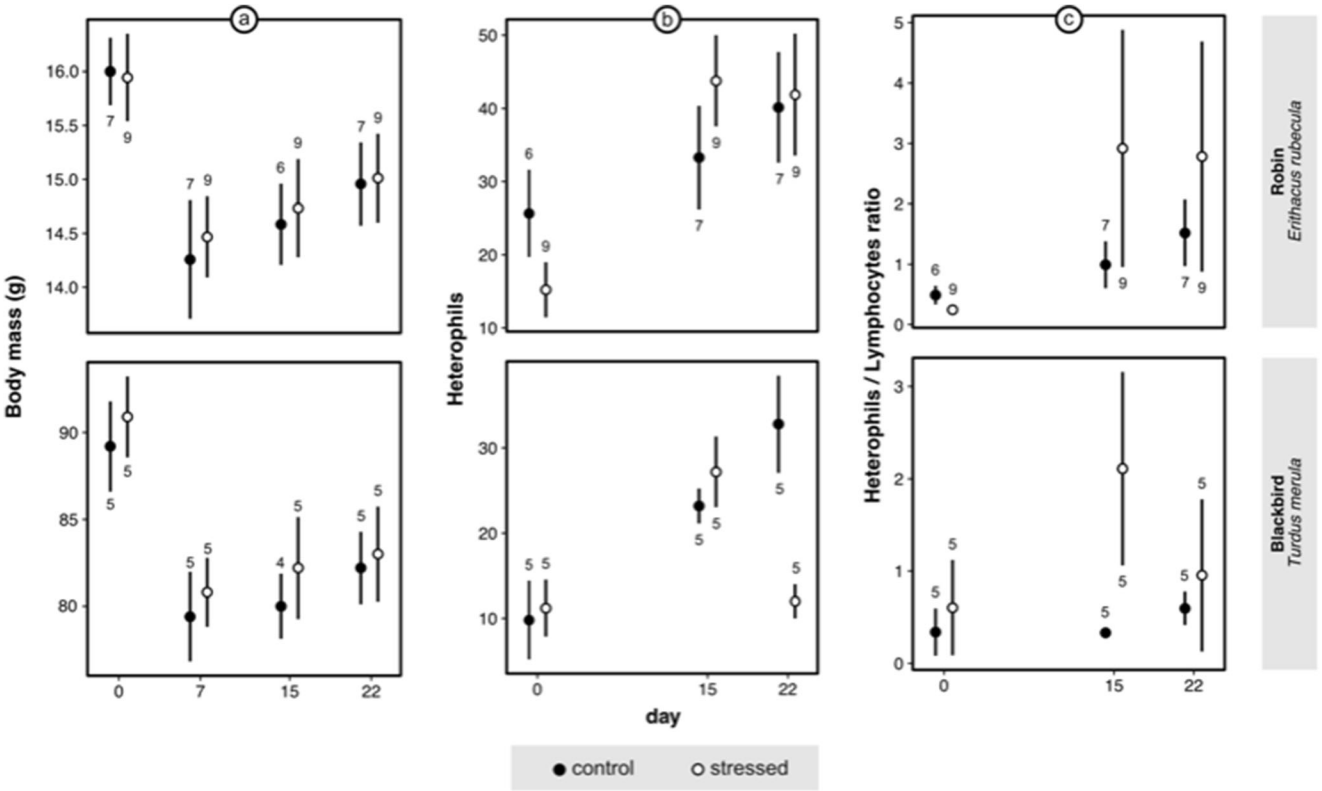

Fig. 2 Variation in body mass (**a**), number of heterophils (**b**) and heterophil:lymphocyte ratio (**c**) in of robins *Erithacus rubecula* and blackbirds *Turdus merula* during the experiment. The control group is represented by filled circles and the stress group by open circles. Values are means ± SE. Number near bars represents sample size

Reliability of Methodologies to Assess *Borrelia* Infection Status

When comparing the performance of *Borrelia* infection diagnostic tests, and considering both bird species, at first sampling (T0), nine birds were infectious to xenodiagnostic larvae, but none of them had infected skin or infected blood samples. At the final sampling (T1), only one of the nine infectious birds had a positive skin biopsy, but five had positive blood samples. At this time, there were six birds with positive blood samples that did not produce positive xenodiagnostic larvae. Therefore, the sensitivity of both skin biopsy and blood analyses were relatively low, varying between 0 (T0) and 0.55 (T1). However, specificity of skin samples was 0.88, whereas that of blood samples varied between 0.64 (T0) and 1.00 (T1; see Online Resource 3). Symmetry and agreement statistics revealed that there was no agreement between xenodiagnoses and analysis of skin biopsies at initial sampling (initial sampling: $\chi^2_{1,23} = 4.45$; $P = 0.035$), but this disagreement decreased at the end of the experiment (skin: $\chi^2_{1,25} = 3.6$, $P = 0.06$). At the end of the experiment, there was agreement between xenodiagnoses and blood samples (blood: $\chi^2_{1,25} = 0.4$; $P = 0.53$; see Online Resource 3).

Discussion

This study allowed the experimental evaluation of the impacts that stressors may have on avian physiology and infection dynamics, in particular, avian hosts' *Borrelia* infectivity to vector ticks. This can thus serve as a model to help to understand how environmental stress may affect pathogen transmission. The physiology of wild-captured birds that were maintained in captivity showed a marked variation with time in this experiment. This was likely caused by the stress induced by the introduction and maintenance in captive conditions, as shown by the steep decrease in bird body mass between day 0 and day 7 (i.e. before the initiation of the stress protocol) and possibly related with changes in behaviour such as difficulties to adapt to the available food items and increased activity. In wild-caught chukars (*Alectoris chukar*), weight significantly decreased right after capture and transport into captivity, and [48] suggested that a state of chronic stress was induced by these actions, and birds only began to recover within 9 days of capture.

At a finer scale, we could detect an effect of the stress protocol on the number of circulating heterophils: (a) in robins, the increase in circulating heterophils was especially evident in the stressed group; and (b) in blackbirds, although both control and stressed groups increased heterophil numbers

Table 2 *Borrelia burgdorferi* s.l. infection in xenodiagnostic tick larvae and tissues from robins *Erithacus rubecula* and blackbirds *Turdus merula* according to sampling periodand treatment

	Robins						Blackbirds					
	Control			Stress			Control			Stress		
	T0	*n*	T1	*n*	T0	*n*	T1	*n*	T0	*n*	T1	***n***
Borrelia prevalence in xenodiagnostic ticks (%)	2.04 ± 2.04	7	2.04 ± 2.04	7	0.00	9	1.59 ± 1.59	9	42.85 ± 17.15	5	45.71 ± 17.15	5	42.85 ± 17.15	5	45.71 ± 17.15	5
Mean estimated *Borrelia* genome copies in xenodiagnostic ticks	0.01 ± 0.01	49	0.01 ± 0.02	49	0.00	63	0.01 ± 0.01	63	3486.00 ± 2164.35	35	2168.00 ± 782.63	35	1261.00 ± 923.57	35	328.00 ± 145.52	35
Borrelia prevalence in blood (%)	na	0	28.57 ± 18.44	7	na	0	33.33 ± 16.67	9	0.00	5	60.00 ± 24.49	5	0.00	5	60.00 ± 24.49	5
Borrelia prevalence in skin biopsies (%)	0.00	6	0.00	7	11.11 ± 11.11	9	11.11 ± 11.11	9	0.00	5	20.00 ± 20.00	5	20.00 ± 20.00	5	20.00 ± 20.0	5

na non available data, *n* samples tested

T0 sampling period before exposure to a stress protocol in captivity, *T1* sampling period after exposure to a stress protocol in captivity.

during exposure to stress protocol, heterophils decreased steeply after the stress protocol ceased in the stressed group only, suggesting that the relief from the stress treatment was particularly beneficial for the blackbirds. This is because, in response to the activation of the hypothalamic-pituitary-adrenal (HPA) axis when an organism is exposed to stressors, the release of glucocorticoids stimulates an influx of heterophils into the blood stream from the bone marrow and reduces their outflow from the blood to other compartments [49, 50]. The number of heterophils may also be affected by infection relapses and associated inflammatory processes and phagocytosis, but no significant effects of infection on the number of circulating heterophils were detected (see below). The generalist stress indicator H/L ratio increased in robins along the experiment but with no evidence of any effects of exposure to the stress protocol. The evaluation of other stress indicators such as corticosterone would have been useful for a better understanding of the efficacy of the stress protocol, but limitations in blood sample volume precluded these analyses.

One could expect that the levels of haematocrit would be reduced by the introduction into captivity [48] and during the course of the experiment due to blood sampling and tick feeding. However, there was no significant variation in the haematocrit during the course of the experiment. This suggests that there was no major deleterious effect of blood depletion in birds, unless the increase of large immature erythrocytes in circulation, masked any effects of sampling and tick feeding on blood oxygen carrying capacity [51].

In our study, there was a tendency for infectious agents (haemosporidians and *Borrelia*) to co-vary. Because the relationships between immune indices and infection intensity in hosts may be affected by interacting co-infections, these should be accounted for in these types of studies [52]. Biard et al. [52] showed that blackbird intestinal parasites explained a significant part of the variance in most immune indices (leucocyte profiles, erythrocyte sedimentation rate, haematocrit) through their interactions with other parasites (*Plasmodium* sp., feather mites), suggesting that these parasites, together, have a strong influence in modulating immune function [53]. In our study, there was a positive association between haemosporidians and WBC in blackbirds, suggesting that malaria infection triggered an increase in immune system activity [53], but we did not detect any significant interactions between malaria and *Borrelia* affecting other physiological indices. Malaria prevalence detected in this study (100%) was similar to that previously reported for *T. merula* in Iberia (93%; [45]) and France (97%; [54]) but lower than that reported for the Azores archipelago (50–60%; [55]). Generally, robins have a much lower malaria prevalence than blackbirds. For this reason, which would likely limit a robust statistical analysis for this bird species, and because of their small body size and consequent higher

sensitivity to transport, we decided to secure only a minimum amount of blood necessary for the most important parameters to be evaluated in our experiment.

Despite the physiological changes throughout the experiment described above, screening of *Borrelia* infection in xenodiagnostic ticks did not suggest infection reactivation after the stress-inducing protocol, because both prevalence of *Borrelia* and spirochaete load in engorged xenodiagnostic larvae were similar in both groups at the two sampling periods of the experiment. However, *Borrelia* prevalence and spirochete load in xenodiagnostic ticks were significantly higher in those ticks that fed on blackbirds than in those that fed on robins. This is in accordance with blackbirds being considered one of the most important reservoir hosts in *B. burgdorferi* s.l. enzootic cycle [26, 56]. Several studies have shown that different wild host species belonging to the same order may differ in their reservoir competence for *Borrelia* [57–60]. In our study, three robins successfully transmitted *Borrelia* to xenodiagnostic larvae, but the prevalence of infection in ticks that fed on these individuals was low as was their spirochaetal load. This is in accordance with the very occasional reports of *Borrelia*-infected larvae collected from this species [28, 29], and further suggests that robins may act as *Borrelia* transient reservoirs only [19]. Some genospecies and strains in a given host are unable to bind to the vasculature and their continued circulation in the bloodstream leads to bacterial lysis by complement components or uptake by circulating phagocytes [61]. These mechanisms of infection and tissue tropism are modulated by the interaction between genospecies and host species [62]. Depending on these interactions, after the tick bite, *Borrelia* infection may persist in the skin and be accessible to the next biting tick, and/ or disseminate to internal organs where it can be sequestered in immune-privileged areas [63].

Because robins were exposed to the crowding instead of rolling cart stressor, one cannot discard that this may have affected their infectivity to ticks, because crowding may be considered a milder stressor compared to cage rolling [34]. However, even if this was the case, our results show that the effect of bird species was significant by itself, with no interactions with stress treatment, and the infectiousness of blackbirds was higher than that of robins also before the stress treatment. Curiously, one of the robins developed a foot joint inflammation during the experiment (approximately 2 weeks after capture). This is an often reported symptom of *B. burgdorferi* s.l. infection in susceptible hosts such as some strains of mammal laboratory models [64] caused by the migration of spirochetes to the connective tissues surrounding joints. This causes inflammation to develop in the joint

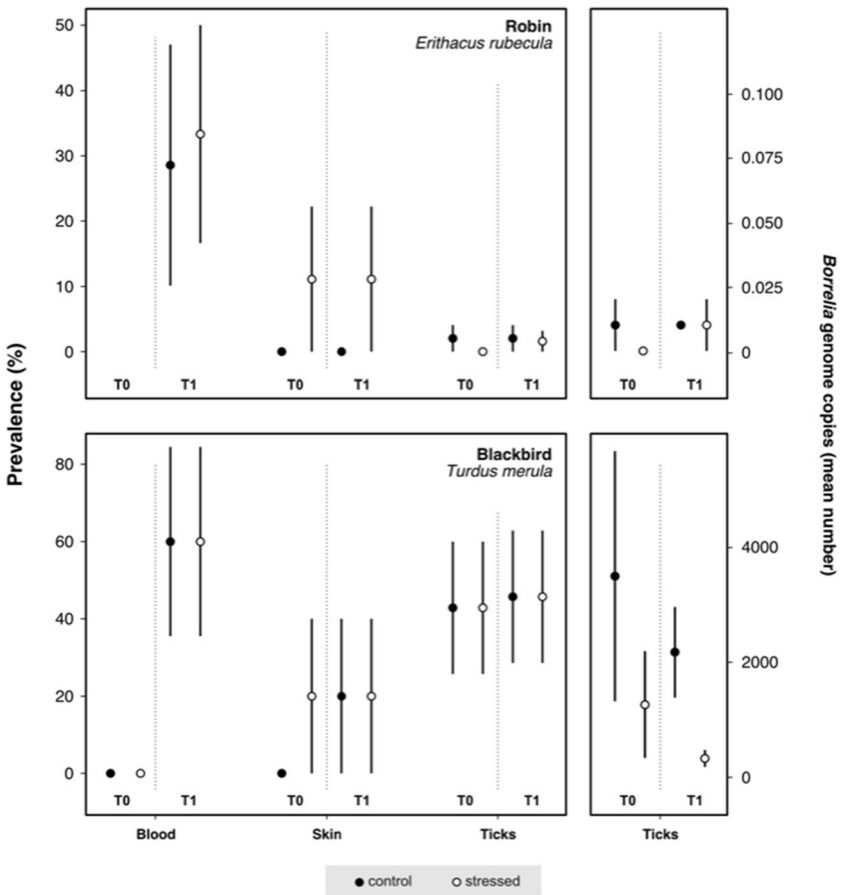

Fig. 3 Variation in prevalence of *Borrelia burgdorferi* s.l. infection in blood, skin biopsies and xenodiagnostic ticks—left axis; and spirochaete load in xenodiagnostic ticks—right axis, in robins and blackbirds at first (T0) and at final sampling (T1) (see Fig. 1 for further details). The control group is represented by filled circles and the stress group by open circles. For sample sizes, see Table 2. Values are means ± SE

capsule, followed by fibrin deposition, as well as increased synovial hyperplasia [65, 66]. We also noticed a high number of heterophils in the smear of the nodule aspirate, but because we did not detect *Borrelia* DNA in the aspirate collected from the nodule, it was not possible to confirm whether this was a symptom of infection.

Although we did not observe an increase in infectivity to ticks during our evaluation period, we detected a higher prevalence of *Borrelia* infection in the blood of blackbirds by the end of the experiment when compared to the time of capture. At the end of the experiment, five robins were also found to have positive blood samples. Although infection prevalence assessed by blood analysis and xenodiagnoses did not differ statistically at the end of the experiment, the sensitivity of blood analysis was only 0.55 and the infection status assessed by both techniques for the same individual did not always match (specificity of blood analysis was 0.64). Previous studies suggested that *Borrelia* may not be detectable in the blood, even in infectious birds [13, 20]. Newman et al. [24] reported that 12 wild individual birds that carried infected larvae, either had a different genospecies of *Borrelia* in their blood (3 individuals) or tested negative for blood infection (9 individuals). However, our results could be influenced by the fact that a sequestered infection in latent states in internal organs could have relapsed in the blood stream later during the course of the experiment. Therefore, it may not have been captured by the final xenodiagnosis, in which, most ticks fed 3 days before blood collection. However, infection relapses caused by stress-exposure have been reported to occur approximately 10–15 days after the onset of body mass decrease [16] which is earlier than our final xenodiagnosis.

We recognise that if the birds had been allowed to acclimatise to captive conditions before stress protocol initiation, it would be easier to attribute any changes in physiology or infection dynamics to the stress protocol itself, rather than the stress induced by the experimental procedure as a whole (including the introduction and maintenance of the birds into captive conditions). However, Biard et al. [52] showed that immune indices and parasite burdens did not change in blackbirds during the first 2 weeks after capture and transfer into outdoor aviaries. Introduction in captivity itself has been shown to induce weight loss, decrease haematocrit, alter baseline and stress-induced corticosterone levels and heart rate but also immune function in birds [48, 67–69], although the direction of response has not been always consistent among studies. Love et al. [68] reported that *Passer domesticus* showed delayed wound healing, reduced spleen and liver size but increased bactericidal ability after introduction in captivity, whereas in the study by Martin et al. [70], bactericidal activity decreased with introduction in captivity in the same species, which was suggested to be related with different methodologies to assess bactericidal activity. Knowledge on the effects of captivity on physiology, prevalence of infection and probability of disease transmission are important issues not only to better understand disease ecology, as in our study, but also in the scope of wildlife rehabilitation programmes and captive breeding. Previous studies performing similar chronic stress protocols [34, 35] in wild-caught passerines refer to acclimatisation periods of 3 months. Because body mass had not completely recovered after 22 days of captivity in our study, we suggest that at least 1 month of acclimatisation period before initiation of the stress protocol would be adequate. However, limitations to such long-term maintenance of small passerines in captivity precluded a longer acclimatisation period, especially when the intention was to release them back into the wild before the start of the breeding season. A larger sample size could also improve the interpretation of our results (i.e. concerning potential confounding factors), and allow us to draw more robust conclusions; however, we limited our sample size to a minimum to reduce the number of animals in the experiment. Nonetheless, in our study, one can still evaluate the effect of stressful events on *Borrelia* infection relapses in avian hosts. Our results suggest that stress may reactivate infection, as shown by the increased prevalence of *Borrelia* in the blood stream, but, at least in the timeframe of our study, we did not observe a significantly increased infectivity to ticks, which is, ultimately, the most important factor for the maintenance in the circulation of this vector-transmitted pathogen. Another factor that may influence pathogen acquisition by xenodiagnostic ticks is the host-acquired resistance to tick feeding in subsequent infestations (as those of our consecutive xenodiagnoses; [71]). If resistance to tick feeding is acquired, tick feeding and engorgement, and pathogen acquisition may be compromised [10, 72, 73]. This could be exacerbated in transient reservoir hosts: four out of five robins with positive blood samples were negative by xenodiagnoses, whereas this only happened in two out of six blackbirds. Also, it is important to note that the sensitivity of detecting *B. burgdorferi* s.l. via xenodiagnoses is lower for engorged larvae compared to flat nymphs [74] which could underestimate *Borrelia* infection prevalence and limit the sensitivity of the technique. Diagnosis of infection could also be improved by culturing *Borrelia* in parallel to real-time PCR diagnosis (in xenodiagnostic ticks but also in host tissues) which could increase detection due to increased bacterial load, and genetic material available for sequencing.

In this study, skin biopsies did not accurately reflect infectiousness of the birds showing a lower prevalence than that obtained from xenodiagnoses, and a low sensitivity. This contrasts with another recent study using the same model system [20], but is in accordance with the results from [14, 75, 76] in which infection was more easily detected through xenodiagnosis than skin biopsy analysis in avian hosts. Age of infection has been suggested to influence the utility of skin biopsies as a monitoring technique [20]. Because the birds in our study were captured in the wild, we do not know when they acquired

Borrelia infection. Possibly, the infection in the birds from our study was in a latent state and its intensity, even in the skin, was very low, being undetectable by direct PCR but enough to infect feeding ticks. The infection was only detectable in engorged xenodiagnostic larvae due to the multiplication of spirochaetes in the tick midgut during feeding, becoming more easily detected by PCR [77]. Because tick feeding and tick salivary gland molecules provide an immunologically modified environment in the host that enables the establishment and dissemination of tick-borne infectious agents [72, 78, 79], it may be possible that it also enhances local proliferation of existing latent and mild infections. The topic application of a dermocorticoid could help to further understand the difference in sensitivity between xenodiagnosis and analysis of skin biopsies, which could be related to the local immunosuppressive effect of tick feeding during xenodiagnoses and improve the detection of infection in skin biopsies as shown by Lefeuvre et al. [80]. Also, we cannot discard the hypothesis that infections in skin biopsies caused by stress-induced immunosuppression could be detectable only later during the course of our experiment, resulting from secondary re-infections and via redistribution of spirochaetes in circulation in the blood stream (see above). However, mechanisms of *Borrelia* dissemination in the host, via the blood stream and by tissue migration with skin and connective soft tissues as intermediate media for spirochete spread, are still to be fully understood [81].

Studying the factors that affect natural host ability to disseminate pathogens is essential to understand the epidemiology of infectious diseases in their natural environment. Our pilot study on the effects of stressors on physiology and infection sets a framework for understanding host-pathogen interactions during the stress response. Using *B. burgdorferi* s.l as a model, our study suggests that captivity might reactivate infections as the detectability of *Borrelia* in the blood of blackbirds by the end of the experiment increased, but reservoir capacity and infectiousness to vector ticks are mostly related with bird species. However, this should be verified with experimental setups involving long acclimatisation periods and further sampling points of xenodiagnosis.

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Author contribution ACN, JAR and ILC conceived and designed the study. ACN and PMA performed the experiments and analysed the data. ACN, ILC, LA, HG, SS and RJL collected data and performed laboratory work. All authors contributed to the writing of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Declarations

Ethics approval The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals and were performed under licence of the competent authorities (Licence No. 694/2016/CAPT and No. 10/2017/CAPT).

Consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

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