DOI: 10.7589/JWD-D-20-00218

Journal of Wildlife Diseases, 57(3), 2021, pp. 553–560

ABSENCE OF *BATRACHOCHYTRIUM SALAMANDRIVORANS* IN A GLOBAL HOTSPOT FOR SALAMANDER BIODIVERSITY

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ABSTRACT: *Batrachochytrium salamandrivorans* (*Bsal*) is an emerging fungal pathogen that affects salamander and newt populations in Asia and Europe. In the Western Hemisphere, *Bsal* represents a major threat to endemic amphibian populations, which have not evolved resistance to infection, and which could experience local extinction events such as those observed in European fire salamanders (*Salamandra salamandra*). We report findings of a survey focusing specifically on wild lungless salamanders in the southeastern US, the most biodiverse location for salamander species globally. Between May 2016 and July 2018, we conducted 25 surveys at 10 sites across three ecoregions in Tennessee, US. Using quantitative (q)PCR, we screened water samples and skin swabs from 137 salamanders in five plethodontid genera. Although single replicates of six samples amplified during qPCR cycling, no samples could be confirmed as positive for the presence of *Bsal* with 28S rRNA PCR and independent laboratory screening. It is probable that we found false positive results, as reported by other researchers using the same assay. We offer recommendations for future monitoring efforts.

Key words: Amphibian disease, fungal pathogen, mycosis, salamander conservation.

INTRODUCTION

Over the past 20 yr, emergent mycoses have severely impacted multiple vertebrate taxa, including bats (due to *Pseudogymnoascus destructans;* Gargas et al. 2009) and snakes (due to *Ophidiomyces ophiodiicola;* Allender et al. 2015; Lorch et al. 2015). *Batrachochytrium dendrobatidis* (*Bd*) is a well-studied dermatophytic pathogen that has contributed to anuran population declines on all continents where amphibians exist (Bosch et al. 2001; Garner et al. 2006; Belden and Harris 2007; Scheele et al. 2019). Martel et al. (2013) discovered a congeneric fungal pathogen, *B. salamandrivorans* (*Bsal*), that nearly extirpated a population of endangered fire salamanders (*Salamandra salamandra*) in the Netherlands. It appears that *Bsal* specializes in colonizing skin of salamanders and newts, although it can survive on anuran hosts (Stegen et al. 2017). It has only been found in Asia, where it probably originated, and in Europe, where it recently invaded (Martel et al. 2014; Nguyen et al. 2017). Since its discovery in Europe, *Bsal*'s geographic and host ranges have expanded rapidly and it has caused recorded population declines at 50 sites, mostly in Germany (Lötters et al. 2020).

Chytridiomycosis, the disease caused by infection with *Bd* or *Bsal*, has imperiled more vertebrate species than any other infectious disease known to science (Gascon et al. 2007). It is recognized that *Bsal* poses an enormous threat to naïve North American salamanders, which lack adaptive resistance to infection (Stokstad 2014). The eastern US holds the greatest number of salamander species globally (Order Caudata; $N \approx 101/755$ species; Highton 1995; AmphibiaWeb 2020). The lungless salamander family Plethodontidae alone represents more than 64% of all described salamander species, with 33% (161/491) of these residing in the US (Conant

TABLE 1. Sites within Tennessee, USA, and number of skin swabs from lungless salamanders (Plethodontidae) and water samples taken for the detection of *Batrachochytrium salamandrivorans*.

Site	Tennessee county	No. samples collected
Bridgestone Firestone Centennial Wilderness	White	20 skin swabs, 1 water sample
Catoosa Wildlife Management Area	Cumberland	13 skin swabs, 1 water sample
Frozen Head State Park	Morgan	3 skin swabs
Big Ridge State Park	Union	7 skin swabs
Panther Creek State Park	Hamblen	1 skin swab
Prentice Cooper Wildlife Management Area	Marion	76 skin swabs, 2 water samples
Elkmont	Sevier	7 skin swabs
Cosby Campground	Cocke	9 skin swabs
Road Prong	Sevier	13 skin swabs
Clingmans Dome	Sevier	2 skin swabs

and Collins 1998; AmphibiaWeb 2020). Many salamander species throughout the region are endemic, and they represent ecologically vital components of forest and stream ecosystems (Highton 1995; Niemiller and Reynolds 2011; Gray et al. 2015). For species already threatened by rarity or habitat loss and degradation, a disease outbreak could have drastic impacts on populations.

Brazilian, Asian, and US amphibians have been infected with *Bd* at low to moderate levels for at least 100 yr (Rodriguez et al. 2014; Zhu et al. 2014; Talley et al. 2015); the oldest evidence of *Bsal* has been found in a sword-tailed newt (*Cynops ensicauda*) dating to 1861 (Martel et al. 2014). Thus, both chytrid species coexisted stably in an enzootic state with amphibian populations until the late 1900s. Since that time, biologists have observed rapid range expansions of these pathogens (i.e., to an epizootic state; Farrer et al. 2011; Talley et al. 2015; Stegen et al. 2017). The driving force behind the transition from enzootic to epizootic status is probably the exotic pet trade (Nguyen et al. 2017; O'Hanlon et al. 2018). Conservationists are gravely concerned that *Bsal* might be introduced to the US via the pet trade, and many have called for international trade regulations, monitoring, and development of task forces and response strategies (Stokstad 2014; Gray et al. 2015; Grant et al. 2016). In 2016, the US Fish and Wildlife Service added 201 nonnative salamanders to the Lacey Act list of injurious species, to restrict importation of these species and to reduce the risk of *Bsal* introduction to novel habitats (US Fish and Wildlife Service 2016).

Since efforts to monitor for the presence of *Bsal* in the US began, no positive detections have been found (Bales et al. 2015; Parrott et al. 2016; Klocke et al. 2017; Newman et al. 2019; Hardman et al. 2020; Horan et al. 2020). A recent large-scale study of over 11,000 samples, primarily from free-living newts (family Salamandridae), in Mexico and the US also failed to detect *Bsal* (Waddle et al. 2020). Although some members of the family Plethodontidae are known to be susceptible to infection by *Bsal* (Carter et al. 2020), surveillance of *Bsal* prevalence in plethodontid salamanders in the southeastern US has been lacking. Therefore, the objective of our study was to survey wild plethodontid populations in three physiographic ecoregions of Tennessee, US, for the presence of *Bsal*. Tennessee holds almost a third (45/161; 28%) of all US plethodontid species (AmphibiaWeb 2020).

MATERIALS AND METHODS

We surveyed wild salamander populations and freshwater habitats for the presence of *Bsal* in three state parks and three wildlife management areas in middle and eastern Tennessee, and at four sites within Great Smoky Mountains National Park (Table 1 and Fig. 1). Between May 2016 and July 2018, skin swabs (n=150) were collected from 137 salamanders belonging to the lungless family

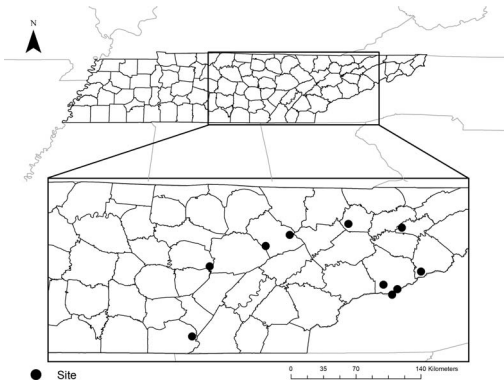

FIGURE 1. Map showing the sites (black dots) in middle and eastern Tennessee, USA, at which lungless salamanders (Plethodontidae) and water samples were surveyed between May 2016 and July 2018 for the presence of *Batrachochytrium salamandrivorans*.

Plethodontidae, specifically, *Desmognathus conanti* (*n*=12), *D. imitator* (*n*=3), *D. monticola* (*n*=9), *D. ocoee* (*n*=1), *D. santeetlah* (*n*=1), *D. welteri* (*n*=1), unknown *Desmognathus* spp. (*n*=60), *Eurycea cirrigera* (*n*=15), *E. longicauda* (*n*=3), *E. wilderae* (*n*=5), *Plethodon glutinosus* (*n*=18), *Plethodon jordani* (*n*=6), *Pseudotriton ruber* (*n*=2), and *Aneides aeneus* (*n*=1). Salamanders were hand-captured and placed individually into clean, numbered, plastic bags, and capture locations were marked with flags. Salamanders were identified to species level using Niemiller and Reynolds (2011). When *Desmognathus* species could not be visually identified due to phenotypic monomorphism (see Wake 1966; Tilley et al. 2008), these individuals were identified to genus level as "*Desmognathus* sp." For sampling, each animal was handled with a new pair of nitrile gloves and transferred from the plastic bag with environmental debris into a clean bag. All animals were thoroughly inspected, and if any clinical signs or lesions were present, photographs were taken, and descriptions were recorded. Salamanders were swabbed in duplicate on all body surfaces for a total of 15 strokes, using a sterile rayon-tipped swab (Puritan Medical Products, Guilford, Maine, USA). Samples were stored in sterile, dry 2 mL tubes and promptly frozen for later molecular analyses. Although most salamanders were swabbed a single time, swabs were collected in duplicate from individuals captured during July 2018, in an effort to increase likelihood of pathogen detection. All salamanders were handled with care and promptly released back to their capture locations, according to institutional, state, and federal permits (IACUC permit no. 15-16—001, IACUC permit no. 19-3002, Tennessee Wildlife Resources Agency permit 3886, Tennessee Department of Environment and Conservation permit no. 2016-026, and US Department of the Interior National Park Services permit GRSM-2016-SCI-1263). Additionally, water samples were collected at four sites, and approximately 500 mL of each sample was vacuum-filtered through a sterile 0.2 µm membrane (Nalgene™ analytical test filter funnel, Thermo Scientific, Waltham, Massachusetts, USA) using a peristaltic pump. To prevent the potential spread of amphibian pathogens between animals and sampling sites, disinfection protocols included 1) disposal of used gloves, plastic bags, and other contaminated refuse in a biohazard bag after each animal had been swabbed, 2) autoclaving of biohazardous wastes, and 3) disinfection of gear and boots with 2% chlorhexidine gluconate.

Swab tips (*n*=150) and membrane filters from water samples (*n*=4) were transferred to cell lysis tubes using flame-sterilized forceps and scissors, and four sterile, 2.3 mm silica beads were added aseptically to each tube. Total genomic DNA was extracted from skin swabs and membrane filters using Omega Bio-Tek Universal Pathogen kits (Omega Bio-Tek, Inc., Norcross, Georgia, USA). Yields of DNA from swabs ranged between 3.7 and 69.1 ng/µL. These yields were comparable to those obtained with the Qiagen DNeasy PowerSoil kit (Leys 2017). Extraction procedures followed manufacturer protocols, with slight modifications to account for swab soak-up of liquids and to increase DNA yield: at Step 3, 300 µL of SLX-Mlus buffer was added to each tube; during the homogenization step, the tubes were shaken with a Digital Disruptor Genie DD38 (Scientific Industries, Inc., Bohemia, New York, USA) at 3,000 rpm for 15 min; from each sample, DNA was eluted to a volume of 100 µL. During individual extraction procedures, DNA isolation blanks were run to control for contamination and false positives.

To validate efficacy of DNA extraction procedures, living *Bd* zoospores were harvested from a 1% tryptone agar plate inoculated with isolate JEL423, purchased from the Collection of Zoosporic Eufungi at the University of Michigan (Ann Arbor, Michigan, USA). Briefly, a swab was streaked across the agar surface that contained active thalli, and the tip was aseptically transferred to a cell lysis tube in the same manner that skin swab samples were transferred. Genomic DNA of *Bd* was extracted according to the same procedures used for skin swab samples, quantified with spectrophotometry, diluted to a concentration of 20 ng/µL, and successfully amplified in an end-point PCR reaction using ITS4 and ITS5 primers (White et al. 1990).

Samples were screened for the presence of *Bsal* using a quantitative (q)PCR assay with primers STeR F and STeR R and probe STeR C defined

by Blooi et al. (2013). Each reaction had a total volume of 15 μL and contained 7.5 μL of 2× Ultra-Fast Brilliant III qPCR master mix (Agilent Technologies, Inc., Santa Clara, California, USA), 0.6 μL 10 μM STeR F primer, 0.6 μL 10 μM STeR R primer, 0.3 μL 1 μM STeR C probe, 3.0 μL sterile water, and 3.0 μL template DNA. Because DNA from a living culture of *Bsal* was not available for use as a positive control, a 180 base pair (bp) synthetic sequence (gBlocks™; Integrated DNA Technologies, Coralville, Iowa, USA) of the 5.8S region of *Bsal* rDNA, with molecular weight 111066.2 grams per mole, was used. To generate a standard curve and establish a cycling threshold (Ct) value, a serial dilution of the *Bsal* gBlocks was prepared at $2e^{-2}$, $2e^{-3}$, $2e^{-4}$, $2e^{-5}$, $2e^{-10}$, $2e^{-15}$, and $2e^{-20}$ ng/μL. Each diluted gBlocks sample was run in triplicate using the assay described earlier to yield a standard curve (efficiency value=2.507; error=0.475). Because the minimum concentration of gBlocks detected by our assay was $2e^{-10}$ ng/μL (approximate gene copy concentration=1.084 copies/μL) at average cycle 36, we set a Ct cut-off value of 36 cycles (Rutledge and Cote 2003; McMullen and Petter 2014). If a skin swab or water sample amplified after cycle 36, we discounted this result as a false positive, following the suggestion of Pfaffl and Bustin (2004).

The gBlocks fragment of *Bsal* rDNA was screened in triplicate at a concentration of $2e^{-4}$ ng/μL concurrently with swab samples in each assay. Two no-template reactions and extraction blanks from each round of DNA extraction (n=7 total rounds) were screened in each assay to rule out false positive results due to contamination. In addition, three aliquots of *Bd* genomic DNA (extracted using methods described earlier) were included in one of the assays to serve as a negative control for nontarget species amplification. Swab samples were assayed in triplicate on a Roche LightCycler 480 (Roche Diagnostics Corp., Indianapolis, Indiana, USA). The thermal profile recommended by Agilent Technologies for this instrument was used, with polymerase activation at 95 C for 3 min, followed by 45 cycles of denaturation and annealing/extension steps (95 C for 15 s and 60 C for 1 min).

A sample was considered negative for *Bsal* if no exponential phase was observed during the first 36 cycles of qPCR (Ct<36) and extrapolated DNA concentrations fell outside the range of the standard curve. When results for a sample were inconclusive, for example, if only one of three replicates produced amplicons, or if exponential amplification occurred during a later cycle (Ct>36), we attempted to reamplify the sample during subsequent runs, as suggested in Pilliod et al. (2013). The sample was classified as "unconfirmed" if a single replicate reaction produced exponential amplification during the subsequent run (Pilliod et al. 2013). From all samples that produced amplicons, DNA was concentrated to approximately 20 μL using a Savant DNA 120 SpeedVac Concentrator (Thermo Scientific, Waltham, Massachusetts, USA) and rescreened using the above procedures.

Aliquots of these samples were sent to a second laboratory for independent screening in triplicate, following a different 15 μL reaction qPCR protocol (Newman et al. 2019). When rescreening attempts produced no amplicons, the unconfirmed positive samples were classified as negative for the presence of *Bsal*, in accordance with recommendations provided by Thomas et al. (2018). During all assays, the positive control (gBlocks) amplified in triplicate, indicating effective PCR amplification of the targeted marker. The no-template negative control samples and DNA extraction blanks did not amplify in any of the 96-well reaction plates.

To further validate results, all unconfirmed positive samples were screened for the presence of a second *Bsal* marker region, using end-point PCR (Iwanowicz et al. 2017). Primers developed by Iwanowicz et al. (2017), as well as newly developed primers Bsal_LSU_DMWassay1 FWD (5′ - CGA GGC AGC TTT GGG TAT AA - 3′) and Bsal_LSU_DMWassay1 REV (5′ - CTT TCC CTC ACG GTA CTT GTT - 3′), were used to target a 28S rRNA gene region unique to *Bsal* isolate AMFP15/1. Each PCR reaction (25 μL) contained 12.5 μL Amresco Hot Start PCR-to-Gel master mix (VWR), 1 μL (20 μM) of each primer, 5.5 μL sterile water, and 5 μL DNA template. Cycling conditions consisted of 94 C for 3 min, followed by 30 cycles of denaturation and annealing and extension steps (94 C for 30 sec, 55 C for 30 s, 72 C for 1 min), and a final extension of 72 C for 10 min.

The ten salamanders captured during July 2018 which exhibited clinical signs of skin diseases were also screened for the presence of *Bd*. We performed a singleplex qPCR assay for *Bd* (Blooi et al. 2013) using primer and probe combinations with the following 15 μL protocol: 7.5 μL 2× Taqman™ Master Mix (iQ Supermix; Bio-Rad Laboratories, Hercules, California, USA), 1 μL each primer (10 μM), 0.5 μL probe (10 mM), 1 μL sterile water, and 4 μL sample DNA. Reaction temperatures were as follows: 50 C for 2 min, 95 C for 10 min, and 45 cycles of the following: 95 C for 15 s; 60 C for 1 min. Each 15 μL reaction was performed in triplicate in a 96-well plate using the QuantStudio™ 5 Flex Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Each assay included a positive control from genomic DNA of cultured *Bd* zoospores (strain JEL197) and a no-template control.

RESULTS

Six swab samples amplified in one out of six reactions run in triplicate on two separate plates (1/6 amplifications for six samples; Supplementary Material Table S1). The second laboratory that independently screened these samples used a different qPCR protocol (Newman et al. 2019) and did not confirm them as true positives (0/3 amplifications for all six samples). Cross-contamination was not a likely cause for positive detections, because none of the no-template or DNA extraction blank controls amplified on any of the tested plates. According to criteria in Thomas et al. (2018), we concluded that *Bsal* was not present on salamander skin or in the water at any of the sites tested. See Table S1 for all metadata, including salamander host species, sample collection locations, dates of sample collection, the number of positive detections per sample, and Ct of amplification curves.

During field work, inspection of animals for clinical signs was not a reliable method for predicting presence or absence of chytrid fungi. Ten salamanders with cutaneous lesions were observed (Fig. 2), but none of these individuals tested positive for *Bd* or *Bsal* using qPCR. Attempts to culture the pathogens by streaking toe clips and swabs from lesions on tryptone-gelatin-hydrolysate-lactose plates (as in Longcore et al. 1999) were unsuccessful. We concluded that neither *Bd* nor *Bsal* were the causative agents of, or contributed to, the disease signs in affected individuals. Further, lesions presented similarly to reports of mite parasitism in plethodontids (Bakkegard et al. 2019; Fig. 2), and we concluded that mites were probably the primary cause of disease.

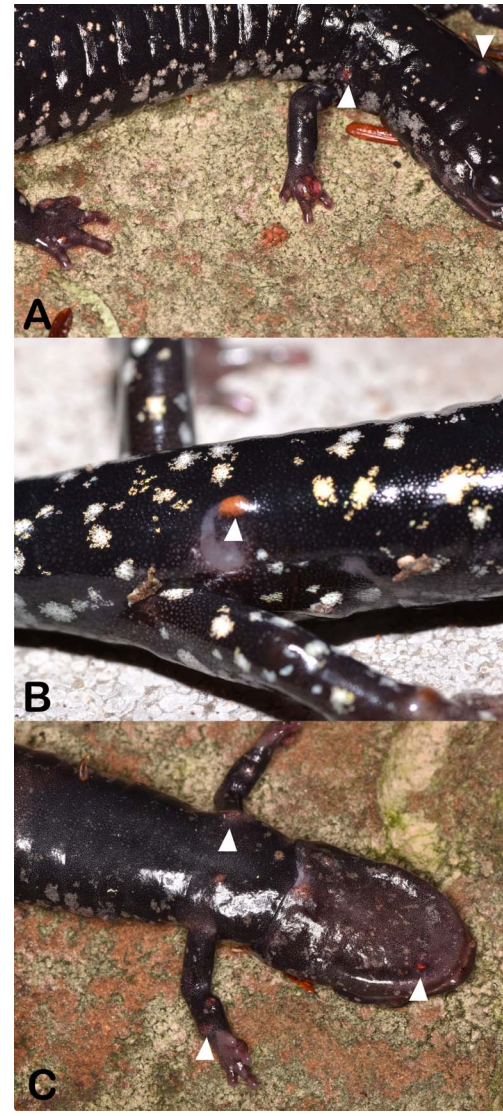

FIGURE 2. Photographs of an individual free-living Northern slimy salamander (*Plethodon glutinosus*) from Tennessee, USA, found with several skin lesions consistent with mite infestation. Lesions were multifocal (approximately 20 total), red-orange raised areas of skin (arrowheads) throughout the body surface but with a majority clustering over the feet and toes. The front right foot had a particularly high burden where several raised areas coalesced, and with an associated loss of a digit. This and other individuals with similar lesions were negative for both *Batrachochytrium salamandrivorans* and *B. dendrobatidis*. Although histopathology was not performed, gross signs paired with PCR results support mite infestation as a likely cause for the observed disease.

DISCUSSION

This work, in conjunction with other studies (e.g., Waddle et al. 2020), further supports that *Bsal* is not currently present in salamander populations of the southeastern US and highlights the importance of developing a thorough diagnostic protocol for future surveillance studies. Molecular techniques such as qPCR assays present diagnostic challenges.

Samples that yield low DNA concentrations (i.e., fewer than 100 copies of the target region) can result in inconsistent results across qPCR cycling runs (Ellison et al. 2006), and limit detection in skin swabs, water, and soil samples. Thus, uncertainty exists regarding cut-off criteria for determining whether a sample is positive. We observed several cases in which only one out of three qPCR reactions produced a positive result (Table S1). One sample also produced a single positive result in a subsequent reaction. Inconclusive outcomes such as these are treated differently by different researchers. Pilliod et al. (2013) state that reamplification of one or two replicates during subsequent qPCR runs (as we found in the single swab sample) is considered a positive detection. However, Thomas et al. (2018) deem such findings as negative. We used conventional PCR of the 28S rRNA gene region as a secondary assay to confirm questionable qPCR results, and did not successfully amplify or sequence *Bsal* DNA. Due to concerns raised about potential false positive results in *Bsal* qPCR assays (Iwanowicz et al. 2017) and inconsistent results (Sabino-Pinto et al. 2018), we adopted a conservative approach to interpretation of our inconclusive results, and described them as negative.

During our study, most of the observed exponential curves occurred after our established Ct cut-off value, and these might be attributable to nontarget DNA amplification (Muletz et al. 2014). Although genomic DNA extracted from *Bd* zoospores did not amplify when tested using our *Bsal* qPCR assay, spurious DNA amplification of a novel, closely related chytrid species might explain the unconfirmed results. Thus, we recommend the use of a multilocus approach, with collaboration from multiple laboratories conducting independent, blind screening of samples. A newly developed qPCR assay targeting a different marker unique to *Bsal*, perhaps with the primer pair developed by Sabino-Pinto et al. (2018) that detects a longer fragment of the internal transcribed spacer gene, should be used to corroborate any positive detections found with the Blooi et al. (2013) assay. Further, qPCR products from all putatively positive samples should be Sanger sequenced and compared to *Bsal* reference sequences. We also recommend attempting fungal culture from any suspicious lesions at the time of sampling, as in Longcore et al. (1999). Ideally, histopathology should also be routinely performed in animals with signs concurrent with *Bsal* chytridiomycosis, to confirm results from molecular protocols.

Preventing *Bsal's* arrival to North America is undisputedly the most effective disease management strategy. Continuation of the US Fish and Wildlife Service restrictions on international trade of exotic salamander species, and expansion of the injurious species list to include anuran species which can serve as vectors for *Bsal* according to Stegen et al. (2017), should be implemented before *Bsal* reaches the global hotspot for salamander diversity. It will be essential for researchers, wildlife managers, and disease response teams to urgently investigate the potential threat that this pathogen poses for North American salamanders and to develop additional diagnostic techniques.

ACKNOWLEDGMENTS

The authors would like to acknowledge the following individuals for helping collect specimens or participating in lab work: Robert R. T. Paine, Brandon Edelbrock, Daniel Istvanko, Isabel Papraniku, Calvin Hall, Robert Buck, Dylan Peters, Gabrielle Russell, Fantasia Erdman, Gabriel Kindl, Andrew Moore, John Griffith, Bailey Burns, Jacob Leys, and Megan Leys. We thank Louise Rollins-Smith and Laura Reinert for providing access to *Bd* culture for positive controls. This research was funded by a US Fish and Wildlife Service grant to DMW (grant 58209).

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-20-00218.

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Submitted for publication 1 December 2020.
Accepted 8 March 2021.