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SHORT COMMUNICATION

Isolation of *Naegleria lustrarea* n. sp. (Excavata, Discoba, Heterolobosea) from the feces of *Ambystoma annulatum* (Ringed Salamander) in Northwest Arkansas

Brian M. Becker[1] | **Idan Banson**[2] | **James M. Walker**[1] | **Anant Deshwal**[3] | **Matthew W. Brown**[2] | **Jeffery D. Silberman**[1,4]

[1]Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, USA

[2]Department of Biological Sciences, Mississippi State University, Mississippi State, Mississippi, USA

[3]Department of Biology, Bradley University, Peoria, Illinois, USA

[4]Biology Center CAS, Institute of Parasitology, Ceske Budejovice, Czech Republic

Correspondence
Brian M. Becker, Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA.
Email: bmbecker@uark.edu

Jeffery D. Silberman, Biology Center CAS, Institute of Parasitology, Branišovská 1160/31, 370 05 Ceske Budejovice, Czech Republic.
Email: jdsilberman01@gmail.com

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Abstract

The salamander, *Ambystoma annulatum*, is considered a "species of special concern" in the state of Arkansas, USA, due to its limited geographic range, specialized habitat requirements and low population size. Although metazoan parasites have been documented in this salamander species, neither its native protists nor microbiome have yet been evaluated. This is likely due to the elusive nature and under-sampling of the animal. Here, we initiate the cataloguing of microbial associates with the identification of a new heterlobosean species, *Naegleria lustrarea* n. sp. (Excavata, Discoba, Heterolobosea), isolated from feces of an adult *A. annulatum*.

KEYWORDS
amphibian, amphizoic, endobiont, eukaryotic gut-microbiome, FLA – free-living amoeba, Heterolobosea

INTRODUCTION

THERE is increasing interest in amphibian-parasite associations, especially in environmentally stressed populations (Blaustein et al., 2018; Daszak et al., 1999; Kilpatrick et al., 2010). Parasites of amphibians are taxonomically diverse and include fungi, protists, bacteria, viruses, helminths, and arthropods (Densmore & Green, 2007). Current research foci include fungal and viral associations (Fisher et al., 2021) as well as bacterial and fungal interactions, because of their potential to devastate amphibian populations (Becker et al., 2015; Burkart et al., 2017; Shu et al., 2019). However, studies of the biodiversity of eukaryotic microorganisms that are integral members of many amphibian gut microbiomes, either as normal enteric biota, infectious agents or opportunistic pathogens are few (Hegner, 1922; Lobeck, 1940), but will hopefully start to gain traction (Li et al., 2023; Weinfurther et al., 2023). This may be especially important for animals that are sensitive to environmental perturbations.

Studies of the parasites of salamanders in the genus *Ambystoma* are relatively few due to the secretive nature of these animals. *Ambystoma annulatum* (Ringed

Salamander), has a very small geographic distribution; being endemic and restricted to the Ozark and Ouachita Mountains of southern Missouri, northern Arkansas, and eastern Oklahoma. As such, Ringed Salamanders are the least studied member of the *Ambystoma* (Conant & Collins, 1998; Trapp, 1956). They are burying (fossorial) salamanders that live in forested habitats most of the year and typically emerge for a few days to a few weeks during fall, or occasionally winter, rains to migrate en-masse to small fishless ponds to breed (Briggler et al., 2004; Semlitsch et al., 2014; Spotila & Beumer, 1970; Trauth, 2000).

McAllister et al. (1995) conducted one of the first studies on the endoparasites of *A. annulatum*. Although they found one micro-eukaryote, the myxozoan *Myxidium serotinum* (Opisthokonta, Metazoa, Cnidaria), and numerous helminths, no endobiotic protists were reported. During an exploratory survey for *Rosculus*-like amoebae (Schuler et al., 2018), we isolated a novel heterolobosean amoeba within the genus *Naegleria* from *A. annulatum* feces. Its nuclear encoded small subunit ribosomal RNA gene sequence (SSU) is unique and phylogenetic analyses place it in a clade with free-living isolates, raising the possibility that this isolate may be free-living with the capacity to infect and live within an animal host (i.e. amphizoic). Because *Naegleria* taxonomy based on morphological features is problematic, molecular data are most often utilized to erect new species designations (De Jonckheere, 2004). Thus, we propose that this isolate represents a new species.

METHODS

Amoeba isolation and culture

Salamanders were collected using a drift fence and pitfall trap the night of October 27 2015 from a small pond in northwest Arkansas (36°09′28.50″N, 94°16′00.82″W; Figure S1). Salamander collection and handling were conducted under the Arkansas Fish and Game Commission Scientific Collecting Permit #032720152 and the University of Arkansas Animal Welfare Committee Protocol #15059 (issued to BMB). At the pond, a fecal sample was aseptically coaxed from a gravid adult female *A. annulatum* (Figure S1), where she directly deposited it onto a weak malt-yeast agar plate (wMY) (1 L dH_2O, 0.75 g K_2PO_4, 0.002 g yeast extract, 0.002 g malt extract, 15 g Bacto agar) in the middle of a uniform lawn of *Escherichia coli*. Great care was taken to ensure that only feces were deposited on the agar plate. The salamander appeared healthy and was subsequently returned to the pond unharmed. The agar plate was incubated in the lab at room temperature (~22°C) in ambient light. After approximately 24 h, a feeding front was observed on the primary inoculation plate. Once the feeding front was ~2.5 cm from the inoculum, amoebae were resuspended in ~15 µl of Page's amoeba saline (PAS; ATCC medium 1323; Page, 1988) and examined on an Olympus BH microscope using phase contrast optics. Amoebae at the feeding front were then resuspended in 10 µl PAS and inoculated at one end of a thick "s-shaped" streak of *E. coli* on a wMY agar plate for continued growth and maintenance. Serial passages were made after amoebae ate through ~3/4th of each plate's bacteria-streak, yielding a mono-eukaryotic culture.

The ability to transition to flagellates was tested by resuspending amoebae in T25 tissue culture flasks with 7 ml of sterile dH_2O, with commercially available spring water (Crystal Geyser), and with ATCC 802 media, each supplemented with *E. coli*. Pathogenic potential in homeothermic animals was tested by incubating cells grown in ATCC 802 media plus *E. coli* in tissue culture flasks at 37°C.

Light microscopy

Amoebae and cysts from just behind the feeding front were mounted on glass slides in PAS and photo-documented using a Zeiss AXIO Imager.A2 under differential interference contrast optics (DIC). Flagellates were photo documented under DIC on a Zeiss Axioskop 2 Plus connected to a Canon (Huntington, NY, USA) CMOS digital camera (EOS R8, 24.2MP full frame mirrorless). Cell measurements were acquired using ImageJ software (Schneider et al., 2012, http://imagej.nih.gov/ij/). Flagellate swimming speed was calculated through real-time video microscopy under phase contrast optics on an Axiovert 135 connected to an EOSR8 camera. Individual video frames with flagellates swimming across the field of view from one point to another were collected (starting frame and ending frame). The elapsed time between the frames and distance traveled (in µm) was measured using imageJ; speed was recorded as µm per second.

DNA isolation, SSU amplification and sequencing

Actively growing amoebae were resuspended in 50 µl of PAS, pelleted by centrifugation at 1200 rcf for 5 min, resuspended in 50 µl of Quick Extract DNA extraction solution (Lucigen) and lysed according to the manufacture's recommended protocol. The nuclear SSU rRNA gene was amplified using "universal" SSU primers (5′_18! 5′-CTGGTTGATCCTGCCAGT-3′ and 3′_24! 5′- TGATCCTTCTGCAGGTTCACCTAC – 3′), 5 µl template DNA and Q5 2X MasterMix (New England Biolab) in a total volume of 25 µl with cycling parameters of 98°C, 30 s followed by 32 cycles of 98°C, 8 s; 60°C, 30 s; and 72°C, 2 min. The PCR reaction was electrophoresed through a 1% agarose Tris-Acetate gel. The amplicon was visualized with Sybr™ Safe (Invitrogen), cut out of

the gel with a razor blade, placed in a p200 filter pipet tip inside of a 1.5 ml centrifuge tube and purified by centrifugation at 10,000 rcf for 10 min. The liquid at the bottom of the tube containing the amplicon was Sanger sequenced completely in both orientations. DNA chromatograms were visualized, edited, and assembled using Sequencher v5.4 (GeneCodes).

Phylogenetic analysis

Within the program Aliview (Larsson, 2014), the SSU rRNA gene was aligned with 36 other rRNA genes that span the currently known breadth of *Naegleria* SSU diversity using MUSCLE (Edgar, 2004). Ambiguously aligned positions were excluded to yield a final data set comprising 1988 sites. Bayesian and Maximum likelihood (ML) phylogenetic analyses were performed using MrBayes v 3.2.7a on the Cipres web portal (Miller et al., 2010; Ronquist et al., 2012; GTR+G+I model of nucleotide change) and RAxML GUI (Edler et al., 2021; GTR+G model of nucleotide change). Bayesian analysis was run on two independent chains sampled every 500 generation until they converged (i.e. the average standard deviation between chains dropped below 0.01). Convergence was reached after 590,000 generations. The first 25% of trees were discarded as burn-in and a majority rule consensus tree and posterior probabilities were computed from the remaining 1772 trees from both runs. Maximum likelihood node support was assessed with 500 rapid ML bootstrap replicates. The optimum trees were visualized with FigTree v 1.4.4 (Rambaut, 2009) and the genus *Naegleria* was rooted according to separate ML analyses (Figures S3 and S4).

RESULTS AND DISCUSSION

A cyst-forming amoeba was isolated from freshly collected feces of a Ringed Salamander, *Ambystoma annulatum*. Trophic and cyst cells possessed a single nucleus with a large central nucleolus (Figure 1 and Figure S2). The nuclei of trophic cells were ringed with refractile granules. Amoebae moved with eruptive pseudopodia (Videos S1 and S2). A bulbus uroid was occasionally observed as were trailing cytoplasmic filaments (Figure 1A,D and Figure S2D–H). Amoebae infrequently transitioned to fast swimming elongated pear-shaped cells with two equal-length apical flagella after the flasks containing liquid media were vortexed for 15 s. Each liquid media tested yielded flagellates (Figure 1E-G and Figure S2K-V). After transformation, only a few (ca. 50) swimming cells were observed, whereas amoebae were dense (ca. $10{,}000^{+}$ cells) and attached to the bottom of the flasks. The nucleus was located at the cell's anterior, and appeared to be associated with the flagellar apparatus. A contractile vacuole was seen at the cell's posterior end (Figure 1E,F and Figure S2). The predominant swimming motion was linear with abrupt directional changes mediated by a slight wiggle of the cell's posterior end or with a very slow rotation (Video S3). Swimming cells moved rapidly, traveling 75–113 μm per second (mean 92 μm per second ($n=6$, sd = 13)) (Video S4). The swimming motion of these flagellates was very different from the more linear and fast rotational motion (like a tight spiral of a well thrown American football) of swimming *Naegleria gruberi* cells (Preston & King, 2003). Cysts formed on agar plates well behind the amoeba feeding front. They were spherical and smooth. Excystment pores could sometimes be seen (Figure 1H,I) but could not be fully enumerated. Consistent with these morphological traits, rooted phylogenetic analyses based on SSU placed it (with full support) within a clade comprising *Naegleria* spp. (Figures S3 and S4), specifically in a robust clade with *N. chilensis* and *N. koreanum* (Figure 1J). These two species are the only two other cultured isolates in this clade and laboratory efforts to induce flagellate transformation consistently failed, despite multiple methods and efforts (De Jonckheere et al., 2001; Khwon & Park, 2018). This clade, together with a clade comprising two *Naegleria* spp. isolated from fish and the clade containing *N. folweri* and *N. lovaniensis*, branched basally within the genus *Naegleria* (Figure 1 and Figures S3 and S4). Because this larger assemblage contains both free-living and opportunistic pathogens, the phylogenic placement of this new isolate raises the possibility that it too may be amphizoic. This is further supported by the observation that an environmental DNA originating from a compost pile yielded a 635 bp SSU fragment (GB# KC164228) identical in sequence to our fecal-derived isolate. A 392 bp ORF-lacking group I intron is present in the SSU gene of our new isolate that is absent in its closest relatives. On the basis of morphology, habitat, and SSU sequence divergence (15 nucleotide differences over 1970 mutually present positions, not including the group I intron, compared to its closest named relative, *N. koreanum*), we propose a new species, *Naegleria lustrarea* n. sp.

Naegleria spp. are typically considered to be free-living amoebae (FLA) that are commonly found in terrestrial and fresh water aquatic habitats (De Jonckheere, 2014). In addition, a number of *Nagleria* species have been isolated from animals, and are known opportunistic parasites. For example, *Naegleria* spp. can cause systemic infections and colonize various organs in fish (Dyková & Lom, 2004). The most well-known species, *N. fowleri*, is an opportunistic pathogen that is responsible for lethal meningoencephalitis in humans and other animals (Henker et al., 2021; Jahangeer et al., 2020; Lozano-Alarcón et al., 1997). Other *Naegleria* species (e.g. *N. italica*, *N. australiensis*, *N. philippinensis*) have been demonstrated to be pathogenic in nature or in experimentally infected animals (De Jonckheere, 2004; Milanez et al., 2022; Scaglia

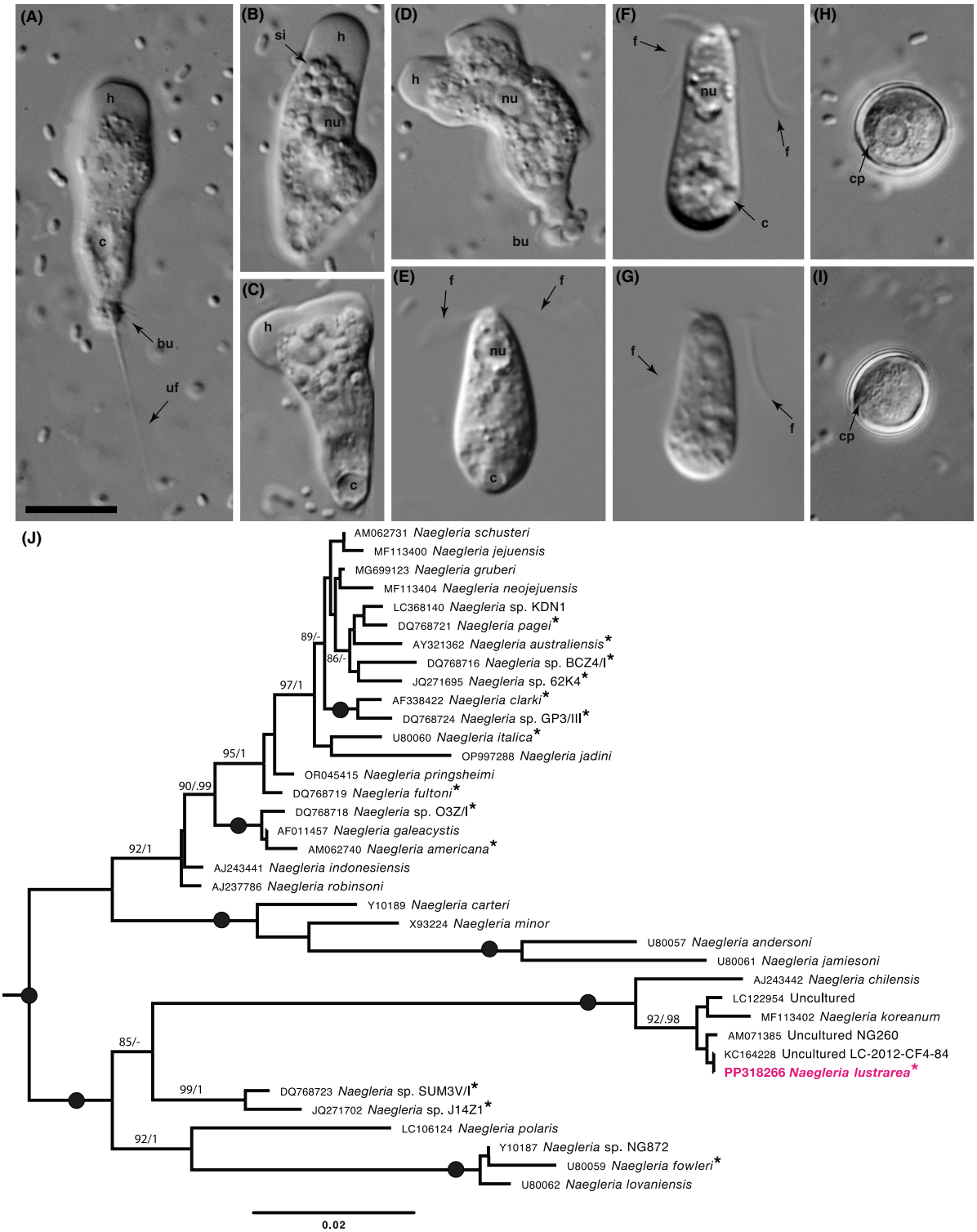

FIGURE 1 Light micrographs and phylogenetic placement of *Naegleria lustrarea* n. sp. (A–D) General appearance of amoebae showing eruptive hyaline pseudopodia (h), bulbus uroid (bu), uroidal filaments (uf), nucleus and large central nucleolus (nu) that almost fills the entire nuclear space, contractile vacuoles (c), and numerous cytoplasmic spherical inclusions (si). (E–G) Biflagellate cells showing two flagella (f) and a single anterior nucleus containing a large nucleolus (nu). (E,F) When visible, the contractile vacuole is at the posterior end of flagellates (c). (H and I) Uni-nuclear (n) cysts with smooth cell wall and cyst pores (cp). DIC optics, and scale bar = 10 μm for all images. (J) An unrooted maximum likelihood phylogenetic tree inferred from 36 *Naegleria* spp. SSU rRNA gene sequences using GTR + G model annotated with node support of at least 85/0.95 with ML bootstrap values and Bayesian Posterior Probabilities, respectfully. Filled circles represent full ML and Bayesian support. *Naegleria lustrarea* n. sp. and the SSU GenBank accession number are highlighted. The root placement is based on the ML analyses depicted in Figure S3. The scale bar represents changes per site. An asterisk by taxon names (*) denote species or isolates known to be host associated.

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et al., 1989). Amphozoic/pathogenic *Naegleria* spp. are scattered across the SSU phylogenic tree (Figure 1J), which raises the possibility that additional FLA in this genus may share the capability of inhabiting various animal/host environments. If *N. lustrarea* n. sp. is an opportunistic pathogen, it poses little threat to warm-blooded animals because it is incapable of surviving more than 6 h at 37°C. We did not directly observe salamander feces microscopically, and cannot be certain if it contained active *N. lustrarea* n. sp. trophozoites. Because of the rapid appearance of a feeding front on the primary inoculation plate, it is reasonable to argue that active amoebae were likely present in the salamander gut.

Ambystoma annulatum is adapted to terrestrial habitats and only migrate to small ponds for breeding in late fall or early winter (Spotila & Beumer, 1970; Trauth, 2000). This salamander species primarily breeds in ponds that do not contain fish (Briggler et al., 2004; Knutson et al., 2004; Semlitsch et al., 2014). However, the collection-pond had introductions of the invasive Golden Shiner (*Notemigonus crysoleucas*) in the months prior to sampling, which could possibly vector fish-associated parasites to native animals (Gaither et al., 2013; Lymbery et al., 2014; Šimková et al., 2019). Without further investigation, it is difficult to know for certain if *N. lustrarea* n. sp. was already present in the intestine of *A. annulatum* from a terrestrial source, if it was already present in, and acquired from the pond, if it was introduced to the pond by fish, or if it is part of the natural salamander gut biota, or an interloper just passing through. Nevertheless, this study adds to the current understanding of *Naegleria* spp. distribution and potential host-range(s). In these times of human-induced habitat and climate alterations, unprecedented amphibian declines, and nonnative fish introductions, further studies are warranted to tease apart the nature of this amoeba/salamander association, especially considering that *A. annulatum* is a salamander "species of special concern".

TAXONOMIC SUMMARY

Naegleria lustrarea Becker and Silberman

ZooBank ID: urn:lsid:zoobank.org:pub:ABC85CB2-1F5D-42B2-918C-CC77794BDD78.

Assignment: Eukaryota; Excavata; Discoba; Heterolobosea; Tetramitia; Eutetramitia; Vahlkampfiidae; *Naegleria*.

Naegleria lustrarea n. sp.

Diagnosis. Amoebae (trophozoites) with eruptive hyaline front; Occasional presence of bulbus uroid; occasional presence of posterior filaments; granuloplasm often packed with spheroid inclusions; 15.4–39.8 µm (average 25.2 µm, SD = 4.2 µm, $n = 100$) cell length, and 5.1–14.3 µm (average 8.8 µm, SD = 1.7 µm, $n = 82$) wide; large nucleus (average 4.4 µm, $n = 50$) with large, central nucleolus (average 3.1 µm, $n = 50$) in diameter that almost fills the nuclear space; round cytoplasmic inclusions 1.1–2.3 µm (average 1.5 µm, $n = 50$) in diameter; Elongated pear-shaped bi-flagellates that typically rapidly swim with a wiggling motion, but occasionally with a rotational and tumbling manner, length 13–24 µm (average 20 µm, SD = 3.2, $n = 24$) width at the widest point 6.8–10.4 µm (average 8.3 µm, SD = 1.0, $n = 24$), flagella are of equal length and roughly 2/3 of the cell length; flagellates possess an anterior nucleus and posterior contractile vacuole; Spherical uninucleate cysts with smooth cell wall; 9.1–11.9 µm (average 10.5 µm, $n = 20$) diameter; cyst pores present.

Type material. This type culture is deposited in a metabolically inactive state with the Culture Collection of Algae and Protozoa (CCAP 1518/36). This culture is also considered the hapantotype (name-bearing type) of the species, under article 73.3 of the International Code of Zoological Nomenclature (ICZN, 1999).

Type locality. Feces from a Ringed Salamander, *Ambystoma annulatum*, captured at small freshwater pond in Northwest Arkansas, USA (36°09′28.50″N, 94°16′00.82″W).

Gene sequence data. The nearly complete nuclear SSU rRNA gene sequence is deposited in GenBank under the accession PP318266.

Etymology. The species *lustrarea* is a Latin derivative of "to transit", as we do not know if this amoeba is passing through the salamander gut or is a natural, active, inhabitant.

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ORCID

Brian M. Becker https://orcid.org/0009-0007-3044-1447
Anant Deshwal https://orcid.org/0000-0002-5700-9335
Matthew W. Brown https://orcid.org/0000-0002-1254-0608
Jeffery D. Silberman https://orcid.org/0000-0002-2965-9197

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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