Functional preservation and variation in the cone opsin genes of nocturnal tarsiers

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The short-wavelength sensitive (S-) opsin gene OPN1SW is pseudogenized in some nocturnal primates and retained in others, enabling dichromatic colour vision. Debate on the functional significance of this variation has focused on dark conditions, yet many nocturnal species initiate activity under dim (mesopic) light levels that can support colour vision. Tarsiers are nocturnal, twilight-active primates and exemplary visual predators; they also express different colour vision phenotypes, raising the possibility of discrete adaptations to mesopic conditions. To explore this premise, we conducted a field study in two stages. First, to estimate the level of functional constraint on colour vision, we sequenced OPN1SW in 12 wild-caught Philippine tarsiers (Tarsius syrichta). Second, to explore whether the dichromatic visual systems of Philippine and Bornean (Tarsius bancanus) tarsiers—which express alternate versions of the medium/long-wavelength sensitive (M/L-) opsin gene OPN1MW/OPN1LW—confer differential advantages specific to their respective habitats, we used twilight and moonlight conditions to model the visual contrasts of invertebrate prey. We detected a signature of purifying selection for OPN1SW, indicating that colour vision confers an adaptive advantage to tarsiers. However, this advantage extends to a relatively small proportion of prey–background contrasts, and mostly brown arthropod prey amid leaf litter. We also found that the colour vision of T. bancanus is advantageous for discriminating prey under twilight that is enriched in shorter (bluer) wavelengths, a plausible idiosyncrasy of understorey habitats in Borneo.

This article is part of the themed issue ‘Vision in dim light’.

1. Introduction

Light is a basic requirement for any image-forming visual system, yet most animals are routinely active under dark (scotopic) light conditions [1]. Given that vision is essential to survival and reproduction—underpinning central behaviours such as predator avoidance, foraging and mate recognition—many or most animals have traits that enable or enhance vision in darkness [1–4]. This truism extends to nocturnal mammals, but puzzling variation exists in their capacity for cone-mediated colour vision, a trait of negligible utility in darkness; i.e. light levels below the threshold of mammalian cone activation, approximately 0.02 cd m⁻² [5–7]. In consequence, many scholars view the colour vision of nocturnal mammals as being immaterial to their visual ecology.

A problem with this assumption is that it conflates the activity pattern of a species (nocturnality) with vision in darkness, ignoring behaviours under cone-active light conditions such as twilight. A greater appreciation for behaviours under dim (mesopic) light levels may inform the functional ecology of colour vision among nocturnal mammals, a topic we investigate here.
Colour vision is based on the expression of two or more spectrally distinct photoreceptors, and most mammals possess two classes of cone—those expressing short-wavelength (S-) and long-wavelength (L-) sensitive opsins [8]. A common exception to this pattern occurs when disabling mutations accumulate on the S-opsin gene (OPN1SW), resulting in M- or L-cone monochromatic vision, or colour blindness, a phenotype with several independent origins among scotopic-active mammals [9–13]. Some authors attribute degenerate opsins to the relaxation of natural selection under light-impoverished conditions, and view colour vision as a functionless anachronism for all nocturnal mammals [14]. This hypothesis is weakened, however, by systematic variation in closely related species with similar nocturnal behaviours. For example, S-opsin genes were lost in some lineages of bats, but retained for many millions of years in others [15–20]. The enduring preservation of dichromatic vision in some bats is strongly suggestive of one or more adaptive functions [17,18], a premise that informs recent research on nocturnal primates.

Primates are extremely visual mammals [21,22], with numerous derivations of the visual system, including convergent orbits, high concentrations of cones and ganglion cells, and (in haplorhine taxa) extreme cortical magnification of retinal foveal regions [23–26]. The collective function of these traits is to increase visual acuity, but they do so in tandem with colour vision, a trait of striking variation across primates. Some nocturnal primates are M- or L-cone monochromats, whereas others have retained the capacity for dichromatic vision [27–36], a pattern that has been interpreted as a state of evolutionary disequilibrium [14]. Under this view, dichromatic vision is a functionless anachronism for any nocturnal primate. Yet signatures of selection on OPN1SW speak to the adaptive function of dichromatic vision for tarsiers [32] and some nocturnal lemurs (Daubentonia [34], Lepilemur, Aotus, Microcebus [35]), demonstrating that colour vision is compatible with nocturnality even if it is incompatible with darkness.

This apparent paradox has fuelled interest in the visual ecology of nocturnal lemurs and shifted attention away from categorical classifications of darkness. Recent fieldwork has focused on dim-light conditions (twilight, moonlight [37]) and the integration of population genetics with field observations and spectral modelling [38–41]. Here we adopt a similar approach with tarsiers (Tarsius), a nocturnal haplorhine taxon. Tarsiers differ from lemurs in having a macula lutea and a fovea with much higher densities of cone photoreceptors [26], traits that enhance visual acuity and support visually mediated predation in the forest understorey [42]. Adaptations for greater visual sensitivity include hyper-enlarged eyes and orbits [24]. The latter trait has deep antiquity in the genus, occurring in Middle Eocene [43] and Middle Miocene [44] fossils. It follows that nocturnal visual predation is an enduring aspect of tarsier biology, yet some evidence points to a diurnal ancestor in the recent past. For example, the rod cell nuclei have a conventional architecture, a strong diurnal trait [45]; and different dichromatic phenotypes suggest an ancestral M/L-opsin polymorphism [29], a trait that appears to be incompatible with nocturnality.

The dichromatic vision of Sulawesi (T. syrichta) and the Philippine tarsiers (T. syrichta) is based on an L-opsin gene [36], which corresponds with small and intermediate ocular morphologies in the genus, respectively [46,47]. In contrast, the dichromatic vision of Bornean tarsiers (T. bancanus) is based on an M-opsin gene [29] and coupled with extreme ocular hypertrophy [46,47]. The covariation of these visual traits is curious (figure 1), and it motivates two related questions: (i) is there an underlying signature of selection associated with the preservation of colour vision in tarsiers? and, if so, (ii) is phenotypic variation in colour vision better explained by genetic drift or natural selection? To explore these questions and to examine the evolution and visual ecology of tarsiers, we initiated an integrative study of opsin genes and prey colour.

2. Material and methods

(a) Study animals and sample acquisition for genetic analysis

Twelve adult or subadult tarsiers (T. syrichta; figure 2a) were hand-captured in the vicinity of Motorpool, Surigao del Norte, Mindanao, Philippines (9.633 N, 125.55 E) and anaesthetized for

Figure 1. (a) Heinrich Sprankel’s preparation of the eye and brain of T. bancanus [48] illustrates the similar volume of the two structures [46]. The eyes of T. bancanus are therefore enormous, both in absolute size and in proportion to the size of the 120–134 g animal. Polyak [49] concluded that the eye size relative to body size of tarsiers is unsurpassed by any mammal. (b) Geographical distribution of M- and L-opsins in Tarsius. (c) Covariation between M- and L-opsins and orbit size (tarsier illustrations © Stephen D. Nash/IUCN SSC Primate Specialist Group, reproduced with permission; skulls after Musser and Dagosto [47], drawn to scale).
obtained using a 3730 DNA Analyser (Applied Biosystems) at ase and exonuclease I (Affymetrix). Nucleotide sequences were purified using shrimp alkaline phosphatase (RNAlater, Qiagen) from each biopsy sample and amplified the S-opsin gene (OPN1SW) to identify the region of capture location.

(b) Amplification and sequencing
We extracted genomic DNA (DNasea blood and tissue kit, Qiagen) from each biopsy sample and amplified the S-opsin gene (OPN1SW) in two fragments, approximately 2 kb each. To identify the region of OPN1SW, we conducted a BLAST search of the reference genome tarSyr1 (T. syrichta) v. 66.1 in the Ensembl browser. Next, we used MEGA v. 5.0 [53] to align the sequence to the corresponding nucleotide sequence of T. bancanus (GenBank accession no. AB111463.1). Lastly, we used Primer3 [54] to design primers for polymerase chain reactions (PCRs; see electronic supplementary material, table S1).

To measure baseline nucleotide diversity, we also collected nucleotide sequence data from intergenic regions throughout the genome of T. syrichta. To avoid ascertainment bias, we used a random number generator to sample initial gene scaffolds from which we selected a sequence fragment of approximately 1 kb. Subsequent BLAST [55] and BLAT [56] analyses of the human genome sequence (GRCh37/hg19; assembly February 2009) allowed us to omit regions with putative gene identity and regions with highly repetitive elements. We then designed primers for the amplification and sequencing of six nuclear genome intergenic regions (see electronic supplementary material, table S1).

To amplify the OPN1SW fragments and intergenic regions, we carried out PCR using HotMaster Taq DNA Polymerase (5Prime), 10X HotMaster buffer (5Prime), 10 mM dNTPs (VWR) and 20 μM primers in 25 μl reactions. For the OPN1SW fragments, the PCR conditions were set at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 63°C for 30 s and 70°C for 120 s and a final hold at 70°C for 120 s. For our intergenic regions, the PCR conditions were set at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 60°C for 30 s and 70°C for 60 s and a final hold at 70°C for 120 s. All PCR products were purified using shrimp alkaline phosphatase and exonuclease I (Affymetrix). Nucleotide sequences were obtained using a 3730 DNA Analyser (Applied Biosystems) at the Molecular Biology Core Facility, Dartmouth College. All nucleotide sequences were deposited in GenBank (accession nos. KX132093–KX132101). Amplification was incomplete for three of 12 samples, and these were excluded from further analysis. For each of the nine remaining samples, the nucleotide sequences were aligned in SEQUENCHER v. 4.2 (Gene Codes, Ann Arbor, MI) using the OPN1SW sequence of T. bancanus to identify exon–intron boundaries.

(c) OPN1SW and intergenic sequence analyses
To explore the functionality and potential variation of OPN1SW in our sample, we compiled and aligned all exon sequences and translated codons into amino acids. We also examined the critical sites that determine spectral absorbance [57,58]. Estimates of the nucleotide diversity population parameter θ for the OPN1SW gene and each of the six intergenic regions were calculated with two statistics: a sample-weighted estimate based on the number of single nucleotide polymorphisms (SNPs) per site (θN; [59]) and an estimate based on the average number of pairwise differences among sequences per site (θs). The average estimates of θN and θs for the six intergenic regions were weighted by fragment length. For OPN1SW, we examined each estimate of nucleotide diversity for non-synonymous and silent sites (synonymous sites + introns). We also computed Tajima’s D [60] (which compares the values θs and θN as expected neutral values. We simulated 5000 genealogies with no recombination to test how often the observed non-synonymous θs, fit simulated distributions under a standard model of neutrality. Finally, we compared the nucleotide diversity of non-synonymous θs and silent sites (θs) using the θN/θs ratio [34]. A ratio less than 1 would be consistent with purifying selection on non-synonymous SNP variation, whereas a ratio greater than or equal to 1 would suggest either relaxation of functional constraint or positive selection on non-synonymous SNP variation. Coalescent simulations, Tajima’s D, θN and θs estimates were calculated using DnaSP v. 4.1 [61].

(d) Irradiance and reflectance spectra
Tarsiers initiate travel and foraging under twilight [62–67]. We therefore measured the irradiance spectra of downwelling

[Image 108x630 to 222x790]
Some tarsiers increase ranging and foraging activities under moonlight (lunar philia [71, 72]), and some authors have suggested that moonlight could be sufficient for primate cone activation and colour discrimination [34, 37]. We therefore measured the irradiance spectrum of moonlight (91% full) in the understorey habitat of *T. bancanus*. For further details, see Melin et al. [37].

We recorded the reflectance spectra of invertebrate prey at four sites: (i) Motorpool, Surigao del Norte, Mindanao, Philippines (9.633 N, 125.550 E); (ii) Visayas State University, Baybay City, Leyte, Philippines (10.747 N, 124.803 E); (iii) Tangkoko Dua Saudara Nature Reserve, Bitung, Sulawesi, Indonesia (1.566 N, 125.233 E); and (iv) Cabang Panti Research Site, West Dua Saudara Nature Reserve, Bitung, Sulawesi, Indonesia (1.216 N, 100.117 E). The inhabiting tarsiers are *T. syrichta* (sites i and ii), *T. tarsier* (site iii) and *T. bancanus* (site iv). At each site, we collected arthropod prey (mostly orthopteran insects) with sweep nets and by hand in areas where tarsiers were observed foraging. The reflectance spectra of prey and their corresponding backgrounds (mature foliage or leaf litter; figure 3) were measured with a USB2000 spectrometer calibrated against the WSI reflectance standard.

The irradiance spectra of twilight and moonlight were used to estimate the radiance spectrum of prey items and to calculate the relative quantum catch ($Q_i = M/L, S$) of cone photoreceptors. The quantum catches of the S and M/L cone classes were calculated by multiplying the reflectance spectrum of the stimulus, $R_\lambda$, the illumination spectra, $I_\lambda$, the spectral sensitivity function of the $i$th photoreceptor and integrating the resulting spectrum over wavelength (equation (2.1)) [73]. The quantum catches were modelled for both dichromatic phenotypes such that $Q_i$ defines the luminance and $Q_S(Q_B + Q_Y)$ defines the yellow-blueness.

\[
Q_i = \int R_\lambda I_\lambda Si_\lambda \, d\lambda.
\]

To determine the conspicuousness of cryptic prey, we calculated the chromatic and luminance contrasts, or differences, of green prey and brown prey against a background of mature green foliage or leaf litter, respectively. These contrasts were calculated for 60 prey items against a sample of 60 corresponding background leaves, yielding a total of 3600 contrasts for each dichromatic phenotype for green and brown prey. Comparisons of significance were investigated using Welch’s two sample t-tests. The significance for all tests was set at $\alpha = 0.05$.

Finally, we calculated colour contrast comparisons in units of just noticeable differences (JNDs) using approximate cone densities [30] and considering quantum noise (calculated as the sum of neural and receptor noise, and proportional to the Weber fraction and inversely proportional to the intensity of the quantum catches [74]). When the colour contrast of two objects (target and background) produces a value that exceeds the threshold of one JND, the target is considered to be detectable against the background. Cone sensitivity curves were approximations based on an S-opsin $\lambda_{\text{max}}$ of 430 nm [29] and an M/L-opsin $\lambda_{\text{max}}$ of 543 or 558 nm for *T. bancanus* and *T. syrichta*, respectively [14]. Visual modelling, including estimates of cone sensitivity curves, was conducted with the package ‘pavo’ [75] in R v. 3.3.1 [76]. The effects of cone optical density and filtering by the macular pigment and lens were not considered.

**Figure 3.** The reflectance spectra of (a) invertebrate green prey ($n = 65$) and mature foliage ($n = 495$) and (b) invertebrate brown prey ($n = 110$) and leaf litter ($n = 200$). Lines depict the mean reflectance spectrum, whereas shading represents the standard deviation.
of variation at our intergenic regions, both as $\theta_W$ and $\theta_S$, and then tested whether the observed nucleotide diversity at non-synonymous sites is lower than expected under neutral evolution. Under purifying selection, the observed nucleotide diversity at non-synonymous sites is expected to be lower than the simulated distributions, reflecting a history of natural selection removing or reducing non-synonymous diversity. Indeed, we found that the pattern of variation at non-synonymous sites in the S-opsin gene ($\theta_S = 0.041\%$) was significantly lower than expected under neutrality ($p = 0.012$), suggesting purifying selection for retaining OPN1SW functionality.

For OPN1SW itself, we combined the introns and synonymous sites within exons into a ‘silent’ class of variation. Although selection may act on such sites, they better represent our expectation for neutral evolution compared with changes that alter amino acids and protein sequences. We compared the level of genetic diversity between the non-synonymous and silent sites [34]. Under neutrality, the frequency distributions of these two classes of diversity are expected to be similar, yielding a value $\approx 1$ for the statistic $\theta_{SN}/\theta_{SS}$. Values substantially less than or greater than 1 may signify negative (purifying) or positive selection, respectively. Our estimate of $\theta_{SN}/\theta_{SS}$ is 0.64, consistent with a history of purifying selection on non-synonymous mutations (table 2).

Taken together, our analysis indicates the functional preservation of OPN1SW, and therefore, dichromatic vision, in a population of Philippine tarsiers. This finding reinforces an earlier result based on T. bancanus [32] and complements recent studies of nocturnal lemurs (Daubentonia [34], Lepilemur, Avahi and Microcebus [35]). The preservation of colour vision in these nocturnal primates, and not others, is an enduring puzzle, and it motivated our exploration of dim-light conditions and the potential advantages of chromatic discrimination.

### 3. Results and discussion

#### (a) Spectral absorbance of the S-opsin

We sequenced OPN1SW in nine individuals of T. syrichta. The coding sequences were free of indels (insertions/deletions), nonsense mutations and premature stop codons, indicating strict conservation and functional preservation of the gene. Among vertebrates, the spectral absorbance ($\lambda_{max}$) of the S-opsin is determined by residues present at seven sites (46, 49, 52, 86, 93, 114 and 118) [57,58]. Here we detected Val46, Ser49, Ile52, Leu86, Pro93, Gly114 and Ser118, residues that strictly conserve and functional preserve of the gene. The levels of nucleotide diversity at non-synonymous sites were lower than those at intergenic regions ($\theta_S = 0.041\%$ versus $\theta_S = 0.085\%$, respectively; table 2). We also used coalescent modelling to simulate neutral distributions of nucleotide diversity based on the observed levels of variation at our intergenic regions, both as $\theta_W$ and $\theta_S$, and then tested whether the observed nucleotide diversity at non-synonymous sites is lower than expected under neutral evolution. Under purifying selection, the observed nucleotide diversity at non-synonymous sites is expected to be lower than the simulated distributions, reflecting a history of natural selection removing or reducing non-synonymous diversity. Indeed, we found that the pattern of variation at non-synonymous sites in the S-opsin gene ($\theta_S = 0.041\%$) was significantly lower than expected under neutrality ($p = 0.012$), suggesting purifying selection for retaining OPN1SW functionality.

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#### (b) Evidence for purifying selection

Our analysis of nucleotide sequence data from six intergenic regions (3416 bp in total) revealed wide variance in estimates of SNP diversity, with two regions containing no polymorphisms and one region containing four SNPs. We observed similar patterns for SNPs in magnitude and frequency, both for the overall dataset of 3416 bp ($\theta_W = 0.100\%$ and $\theta_S = 0.085\%$, respectively) and for the six regions independently (table 1). We found that $\theta_W$ and $\theta_S$ were significantly different in only one comparison ($p < 0.05$). This difference did not reach significance using a coalescent simulation.

#### (c) Activities during sunset and civil twilight

For tarsiers, sunset and civil twilight (when the solar elevation angle, $\theta_S$, is between $0^\circ$ and $6^\circ$) appear to be important cues for initiating activity. Observations of tarsiers exiting their sleeping sites and initiating travel are strikingly consistent across species, occurring 15 min before sunset [63] or between 18.00 and 18.15 hours (range: 17.45–19.10 hours) [62–67]. In Borneo, Crompton & Andau [64] recorded light levels at the onset of activity (mean: 6.8 lux; range: 1.4–15 lux), demonstrating behavioural synchronization with light conditions that support colour vision. It is perhaps significant that nocturnal lemurs are similarly attuned to prevailing light conditions, but mono- and dichromatic taxa (Phaner and Lepilemur, respectively [35]) initiate activities under much darker conditions [78].

Here we report the spectral composition of civil twilight in the understory of our study site on Leyte, Philippines (figure 2b). We found that T. syrichta initiated travel in the light with a peak of approximately 550 nm, a green colour that is typical of light transmitted through canopy foliage [51]. This spectrum differs from that of Melin et al. [37], who recorded understory irradiance in Sabah, Borneo at 18.50 ($\theta_S = 6^\circ$). Their spectrum contained a broad peak around 450 nm, a decidedly blue colour that is typical of civil twilight in open habitats [51,70,79].

Differences in the spectral composition of understory light can be attributed to differences in canopy openness. Denser canopy foliage will attenuate and filter downwelling light to...
a greater extent (resulting in darker, greener understoreys) [51]; and it follows that systematic variation in canopy openness will exert large effects on the colour and amount of light available to tarsiers. We therefore examined geographical variation in canopy openness to better contextualize differences in their visual systems.

(d) Geographical variation in canopy openness

We examined 150 hemispherical canopy photos from Borneo (Danum Valley and Cabang Panti; n = 99) and the Philippines (our field sites on Leyte and Mindanao; n = 51) and compared per cent site openness. Our results suggest that the forest canopies of Borneo are more open (F_{23,51}, d.f. = 140, p < 0.0001) and more variable (Levene’s test, p < 0.0006) than those of Leyte and Mindanao (figure 4). Although it is premature to characterize large heterogeneous islands on the basis of such limited data, our results support the enduring perception of Bornean canopies as being relatively porous, particularly those habitats dominated by dipterocarp trees [80]. This result also speaks to the differential history of cyclone disturbance across insular Southeast Asia; cyclones seldom reach Borneo, but they visit the islands of Leyte and Mindanao regularly (see electronic supplementary material, figure S1). The effects of cyclones on understory light levels are twofold: initially, there is an immediate increase in the light owing to the removal of canopy vegetation; however, light is soon extinguished by the rapid growth of understory vegetation as it fills vacant space in the mid- and upper canopy [81,82]. Countering this, a history of recurrent disturbance will produce darker understories, and it stands to reason that Philippine tarsiers (T. syrichta) are light-limited relative to Bornean tarsiers (T. bancanus). This conjecture requires testing, but it has the advantage of framing curious morphological patterns, such as the apparent trade-off between relative eye size and ear size in the genus [83] (figure 1). It is tempting to suggest that darker understory conditions favour degenerate eyes and a greater reliance on auditory localization.

(e) Visual modelling results

Our working hypothesis—that geographical variation in understory light conditions has shaped the evolution of tarsier visual systems—has the advantage of generating testable predictions. For example, given that the performance of photoreceptors diminishes as they become photon-noise-limited, the \( \lambda_{\text{max}} \) of opsins could be important for maximizing signal-to-noise ratios in dim light. To test this prediction, we estimated the chromatic contrasts of arthropod prey and calculated JNDS under site-specific twilight conditions and standard moonlight conditions.

We found that most prey–background combinations were well matched chromatically, and that each colour vision phenotype produced distinct distributions of chromatic contrasts in twilight (green prey: \( F_{14,35}, \text{d.f.} = 6283.9, p < 0.001 \); brown prey: \( F_{14,35}, \text{d.f.} = 5611.5, p < 0.001 \); figure 5a). We also detected phenotypic differences in the discrimination of achromatic (luminance) contrasts (green prey: \( F_{5000}, \text{d.f.} = 5428.1, p < 0.001 \); brown prey: \( F_{5000}, \text{d.f.} = 5711.6, p < 0.001 \)). However, only a small proportion of the prey–background contrasts exceeded 1 JND, a theoretical threshold for achromatic discrimination (figure 5b). Under moonlight, we detected no phenotypic differences in achromatic discrimination.
We measured the spectral composition of twilight and moonlight in the understorey habitats of this advantage is uncertain. We measured the spectral composition of twilight and moonlight in the understorey habitats of tarsiers and modelled 7200 prey–background combinations, suggesting that the M-opsin of \textit{T. bancanus} is advantageous for visual predation in the relatively light-enriched understorey habitats of Borneo. Taken together, our findings suggest that the M-opsin gene and hyper-enlarged eyes of \textit{T. bancanus} are coupled, adaptive derivations within \textit{Tarsius}. However, if Borneo was colonized by a small founding population with an M/L-opsin gene polymorphism [36], then fixation of the M-opsin allele by drift [84] is also plausible.

(f) Summary conclusions

Our findings reveal a signature of purifying selection for \textit{OPN1SW}, indicating that dichromatic colour vision confers an adaptive advantage to tarsiers. However, the nature of this advantage is uncertain. We measured the spectral composition of twilight and moonlight in the understorey habitats of tarsiers and modelled 7200 prey–background combinations, finding few chromatic contrasts. At the same time, our models suggest that the colour vision of tarsiers can discriminate some prey under dim light, particularly brown prey amid leaf litter. Our models also suggest that the M-opsin of \textit{T. bancanus} is advantageous for visual predation in the relatively light-enriched understorey habitats of Borneo.
Data accessibility. Sequence data has been deposited on GenBank under accession numbers: KX132093–KX132101. Spectral data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/ dryad.q29c0.

Authors’ contributions. G.L.M. and N.J.D. conceived the study. G.L.M. and P.S.O. conducted field research. G.H.P. designed molecular methods. G.L.M. performed the lab work and ensuing analyses. G.L.M. and N.J.D. drafted the manuscript and all authors were involved in revising drafts. All authors contributed to the overall design and interpretation.

Competing interests. We have no competing interests.

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