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Author for correspondence:
Cheyenne Tait
e-mail: ctait@umass.edu

†Present address: Department of Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA.
‡Present address: Department for Biochemistry, Institute for Biochemistry and Biophysics, Center for Molecular Biomedicine (CMB), Friedrich-Schiller University, Hans Knöll Strasse 2, Jena 07745, Germany.
§Present address: Department of Evolutionary Biology, Integrative Zoology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.

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A reversal in sensory processing accompanies ongoing ecological divergence and speciation in *Rhagoletis pomonella*

Cheyenne Tait[1,†], Hinal Kharva[2,3], Marco Schubert[4], Daniel Kritsch[5,‡], Andy Sombke[5,§], Jürgen Rybak[5], Jeffrey L. Feder[1] and Shannon B. Olsson[2]

[1]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA
[2]Naturalist-Inspired Chemical Ecology, National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bellary Road, Bangalore 560065, India
[3]School of Life Sciences, The University of Trans-Disciplinary Health Sciences and Technology, 74/2, Jarakabande Kaval, Post Attur via Yelahanka, Bangalore 560064, India
[4]Department of Biology, Chemistry and Pharmacy, Institute of Biology, Free University Berlin, Berlin 14195, Germany
[5]Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans Knöll Strasse 8, Jena 07745, Germany

CT, 0000-0002-1851-8911; SBO, 0000-0003-4126-7353

Changes in behaviour often drive rapid adaptive evolution and speciation. However, the mechanistic basis for behavioural shifts is largely unknown. The tephritid fruit fly *Rhagoletis pomonella* is an example of ecological specialization and speciation in action via a recent host plant shift from hawthorn to apple. These flies primarily use specific odours to locate fruit, and because they mate only on or near host fruit, changes in odour preference for apples versus hawthorns translate directly to prezygotic reproductive isolation, initiating speciation. Using a variety of techniques, we found a reversal between apple and hawthorn flies in the sensory processing of key odours associated with host fruit preference at the first olfactory synapse, linking changes in the antennal lobe of the brain with ongoing ecological divergence. Indeed, changes to specific neural pathways of any sensory modality may be a broad mechanism for changes in animal behaviour, catalysing the genesis of new biodiversity.

1. Introduction

Changes in behaviour affecting an animal's mate, food or habitat choice (i.e. moth sex pheromones [1], cichlid coloration [2], frog [3] and cricket [4] songs) are often catalysts for rapid evolutionary change and speciation. Behavioural changes in animals may ultimately be due to changes in the nervous system in the detection and processing of sensory stimuli from the environment. While sensory cues affecting behaviour have been identified for many organisms, the neurological mechanisms underlying food, habitat and mate choice leading to evolutionary divergence and new biodiversity are largely unknown.

Here, we provide evidence for a reversal in olfactory processing of fruit odour cues in *Rhagoletis pomonella* (Diptera: Tephritidae) affecting host plant and mate choice and contributing to rapid divergence into a derived race of apple-infesting flies. *Rhagoletis pomonella* is a model for ongoing ecological speciation [5,6], dating back to the origins of evolutionary theory. In later editions of *On the Origin of Species*, Darwin [7] mentioned 'Mr. B.D. Walsh, a distinguished entomologist' and his theory of 'phytophagic races'. Darwin recognized that many phytophagous insects were host-specific, citing Walsh as the authority on the matter. Indeed, Walsh [8] hypothesized that many phytophagous insect specialists arose in the process of adapting to new host plants,

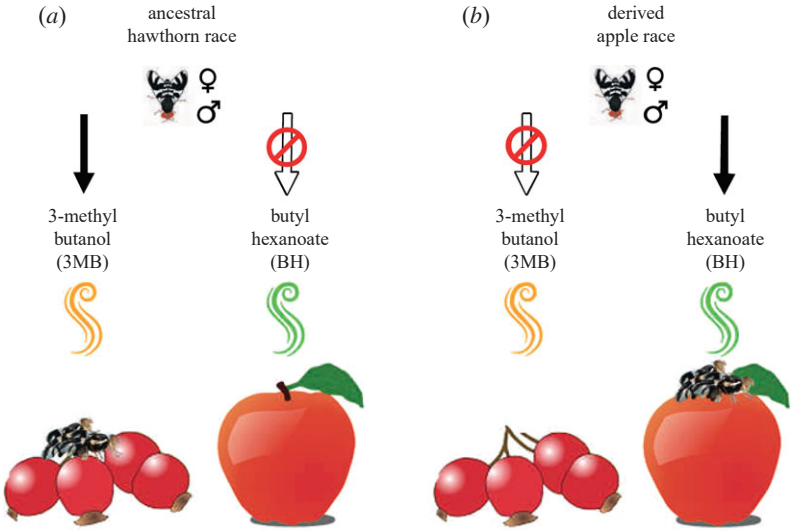

Figure 1. *Rhagoletis pomonella* host fidelity. (*a*) Both sexes of the ancestral hawthorn race are attracted to volatiles from hawthorn fruit. 3MB is essential for hawthorn fly attraction, while BH, a key apple volatile, is an antagonist. (*b*) The same volatiles exhibit opposite valences for the apple race, which uses BH to locate apples and is antagonized by 3MB. The mating flies image was adapted from a photo by Joseph Berger, Bugwood.org, licensed under CC BY 3.0. (Online version in colour.)

claiming as an ongoing example the recently recognized shift of *R. pomonella* from its ancestral host hawthorn (*Crataegus mollis*) to introduced, domesticated apple (*Malus domestica*) in the eastern USA. However, neither Darwin nor Walsh could identify the exact nature of the changes that initiated host shifts and subsequent speciation.

Today, it is thought that a key contributor to insect diversity and phytophagous specialization is that many species, including *R. pomonella*, mate only on or near preferred host plants [5,9]. When mating is host-specific, differences in host choice translate into reproductive isolation, generating a system of positive assortative mating favouring the evolution of additional adaptations affecting survivorship. In the case of *R. pomonella*, after mating, females lay eggs and larvae feed exclusively within the fruit into which they were oviposited. Consequently, host choice in *R. pomonella* is a behaviour that links finding mates for reproduction with finding fruit for oviposition and larval feeding. Adults of both sexes of the ancestral hawthorn and derived apple races initially distinguish between apple versus hawthorn trees using specific olfactory cues [5,10]. Flies display a strong, genetically based attraction to the compounds from natal fruit and avoidance of odours of non-natal fruit [11–13] (figure 1). Thus, coupling of host and mate choice in *R. pomonella*, achieved through an evolved olfactory preference of apple race flies for apple odour, restricts gene flow with the ancestral hawthorn population to approximately 4% [12,14,15]. This reduction in gene flow enabled the divergence of apple flies into a new host race from hawthorn flies within the last 160 years [6,16].

Given the especially rapid formation of the apple-infesting race, the neurological basis for the evolved preference of apple flies for apple odours could involve relatively simple changes in the olfactory system. Synthetic blends of apple and hawthorn fruit volatiles have been developed that differentially attract the host races [12,17,18]. While blends induce maximal behavioural responses, the volatile butyl hexanoate (BH) has been shown to be critical for the apple race's preferential response to apple [12,17]. In comparison, 3-methyl-1-butanol (3MB) is essential for the orientation of the hawthorn race to hawthorns [12,18]. Moreover, when either BH or 3MB is added to the host blend of the other race, the volatile induces avoidance [13,17–19] (figure 1). This system is reminiscent of specific pheromone components in specific ratios attracting and repelling sister species of moths. Additionally, a scenario where attraction and avoidance to a complex plant source is primarily mediated by single volatile components has been reported in mosquitoes in the context of orchid volatiles [20].

We, therefore, hypothesize that changes in processing of the apple and hawthorn-associated volatiles (BH and 3MB) underlie choice behaviours fostering speciation in this system. In insects, volatiles are primarily detected by protein receptors expressed in olfactory sensory neurons (OSNs) in cuticular sensilla on the antennae (figure 2*a*). Previous studies have shown that only two OSN types for each volatile are responsive to either BH or 3MB among sampled OSNs in *R. pomonella*, and these two types are colocalized (figure 2*a*). A change here could impact behavioural responses. However, these neurons are physiologically indistinguishable between apple and hawthorn flies [21,22], exhibiting similar numbers, response profiles and dose–response curves. Thus, the change in host choice displayed by apple flies is not due to change in OSN sensitivity or specificity. From the antennae, OSNs relay information to the antennal lobe (figure 2*b*), where they connect to antennal lobe neurons (ALNs) at neuropil structures called glomeruli. This is consequently the earliest stage in the brain where differential neurological processing may occur. All else remaining the same, a reversal in the processing of BH and 3MB in the antennal lobe would reverse behavioural outcomes, leading to apple race flies having opposite preferences from hawthorn race flies [22]. Such changes in processing in the antennal lobe have been reported in moth pheromone systems [23–26], most notably between the Z and E strains of the European Corn Borer, *Ostrinia nubilalis* (electronic supplementary material, figure S1).

Here, we test for similar change within the antennal lobes of apple versus hawthorn flies in the processing of the behaviourally relevant volatiles BH and 3MB. Our objective is to identify the earliest point of differentiation in the olfactory

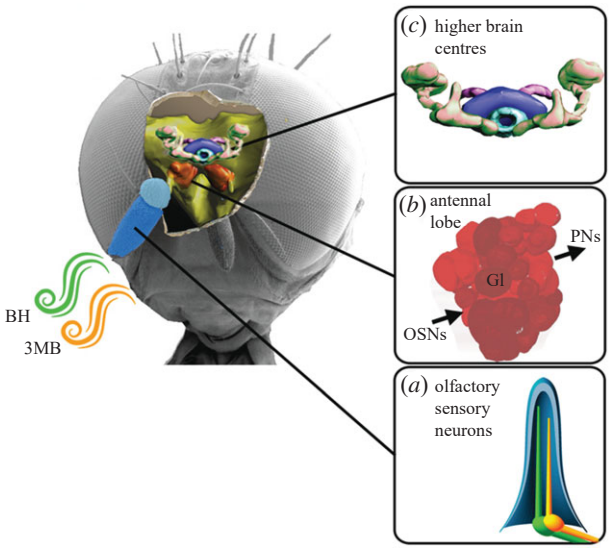

Figure 2. Overview of *R. pomonella* olfaction. (*a*) OSNs are located in stereotyped groupings within sensilla on antennae, where odorants including BH and 3MB interact with protein receptors. (*b*) The antennal lobe (AL) is the first olfactory synapse, where OSNs from the periphery provide input to specific glomeruli (Gl). (*c*) The projection neurons (PNs), a specific type of ALN, bring olfactory information from the AL to higher brain centres such as the mushroom bodies and the lateral horn. (Online version in colour.)

processing pathway between host races that may have played a role in catalysing their rapid evolutionary divergence. To accomplish our goal, we use neurophysiological techniques to trace responses to BH and 3MB from OSNs through the antennal lobe to output via ALNs.

2. Methods

(a) Insects

Apple flies were obtained as pupae from a colony maintained at the NY Agricultural Experiment Station in Geneva, NY, USA, which relocated to the USDA-ARS Appalachian Fruit Research Station, Kearneysville, WV, USA. Additional apple flies and all hawthorn flies were collected as larvae from fruit in Michigan, USA, and reared to adulthood following established *Rhagoletis* husbandry methods [27].

(b) Odours and their delivery

'BH' (Fluka, 98.5% purity), the key component in the apple blend, '3MB' (Sigma Aldrich, 99% purity), the key component in the hawthorn blend, and 1-octen-3-ol (Aldrich, 98% purity), not in either blend, were serially diluted to 10^{-3} w/v in hexane (optical imaging) or mineral oil (optical imaging, intracellular recording, antennal backfilling). They were administered as stimuli on filter paper circles in glass Pasteur pipettes as previously described [22]. Appropriate solvent blanks were also used as controls.

(c) Antennal lobe reconstruction

For brain reconstruction, apple flies were prepared for histology as described previously [28]. Briefly, heads were prefixed 24 h in 80% ethanol, 37% formaldehyde, 100% acetic acid (10 : 4 : 1), washed in phosphate buffer solution (PBS, pH 7.4) and post-fixed 1 h in 2% OsO_4 solution. After dehydration in acetone, heads were embedded in Araldite (Araldite epoxy resin kit, Agar Scientific). Serial sections (1.5 µm) were created with a Microm HM 355 S rotary microtome and stained using 1% toluidine blue and Pyronin G in 1% sodium tetraborate. Sections were imaged with a Zeiss AxioImager Z1 using 10× objective (Plan-NEOFLUAR, 10x/0.3; Carl Zeiss, Jena, Germany).

For antennal lobe reconstruction and immunohistochemistry, flies were prefixed in 4% paraformaldehyde (PFA) for 30 min, dissected and fixed over night at 4°C. After washing in PBS, brains were blocked with 1% BSA for 2 h. Specimens were labelled with monoclonal mouse anti-synapsin 'Synorf1' antibody (1 : 30, provided by E. Buchner, University of Würzburg) and polyclonal rabbit anti-FMRFamide [29] (1 : 2000; ImmunoStar, Hudson, WI, USA) at 4°C for 4 days. After washing in PBS, brains were incubated with Alexa Fluor-488, goat anti-rabbit (1 : 250; Invitrogen Life Technologies, Darmstadt, Germany) and Cy3, anti-mouse (1 : 250; Jackson Laboratories, West Grove, PA, USA) at 4°C for 4 days. Specimens were then washed in PBS and mounted in VectaShield (Vector Laboratories Inc., Burlingame, CA, USA). Z-stacks were scanned in 1 µm intervals at 1024 × 1024 pixel resolution with a Zeiss LSM 710 NLO confocal microscope (Carl Zeiss, Jena, Germany) using a 20× water immersion objective (W Plan-Apochromat 40×/1.0 DIC M27; Carl Zeiss).

For three-dimensional (3D) reconstruction and volume estimation, images from histological methods were aligned and brain structures reconstructed using the segmentation software AMIRA [30,31] (FEI Visualization Sciences Group, Burlington, MA, USA; Advanced 3D Visualization and Volume Modeling, RRID:nif-0000-00262). After surface models were digitized, they were also segmented using AMIRA. Regions of the brain were identified and glomeruli named according to standard insect brain nomenclature [32].

(d) Antennal olfactory sensory neuron backfilling

To identify target neurons for backfilling, we performed single-sensillum recordings from neurons on the antennae, stimulating with BH and 3MB, as described previously [22]. Target OSNs were passively filled for 2 h with 4% neurobiotin (Vector Laboratories, Inc.) [33], then flies were stored at 4°C for 2 h for further tracer diffusion, decapitated and dissected in PBS.

In protocol adapted from *Drosophila melanogaster* [34,35], dissected brains were first fixed in 4% PFA for 3 h on a rotator at 4°C. They were washed with 0.3% Triton X in PBS (PTX) and blocked with 0.1% bovine serum albumin in PTX for 15 min. Brains were incubated with DyLight 488 Streptavidin (1 : 125, Vector Laboratories Inc., USA) and mouse anti-Bruchpilot/mAbnc82 (1:30, DSHB, University of Iowa) to highlight specific neuronal tissues for 48 h on a rotator at 4°C. After washing in PTX, we added goat anti-mouse Alexa 647 (1 : 400, Invitrogen) and incubated for another 48 h on a rotator at 4°C. Samples were washed in PTX at 4°C then mounted in 70% glycerol. Optical sections (512 × 512 pixels) were imaged with a confocal scanning microscope (Olympus FV1000, DSS Imagetech, Bangalore) under a 40×, 1.4 oil-immersion DIC objective, with 1 µm step size. Using the AL reconstruction for guidance, glomerular identification and production of maximum projections was performed in FIJI.

(e) Optical imaging

Mounting and partial dissection of *R. pomonella* to expose the antennal lobe through a window in the cuticle followed methods described for *D. melanogaster* [36,37]. We bath applied 40 µl of membrane-permeant Calcium Green 1-AM ester (Molecular Probes) and Pluronic F-127 in DMSO (Molecular Probes) dissolved to 30 µM in *Drosophila* Ringer's solution [38]. We added 10 µl of MRP transport blocker MK-571 (Enzo Life Sciences) dissolved to 1 mM to prevent neurons from discharging Calcium Green prematurely [39]. The preparation was incubated for 2 h on ice, then rinsed in Ringer's solution. Preparations had to be tilted forward to enable viewing of the antennal lobe around

the opaque cuticle (electronic supplementary material, figure S3). This tilting affected the location of specific glomeruli and influenced ROI (region of interest) choice in later optical imaging analysis.

Imaging was performed using a custom-built system (DSSImagetech, Bangalore, India). A Cairn Research Optoscan-Monochromator (Kent, UK) equipped with a narrow band-pass interference filter (P855/488/N25, Optoscan) was used to excite at 475 nm. An excitation filter (FF01-474, Olympus), a dichroic beam splitter (Di02-R488, Olympus) and an emission filter (BLP01-488R-25, Olympus) allowed the passage of relevant wavelengths. Light emitted was captured by CCD camera (PhotometricsCoolsnap HQ2, Tuscon, AZ, USA) mounted to an upright microscope (Olympus BX51WI) equipped with a water immersion objective (Olympus, 10×/0.30 and 20×/0.5). The system was controlled by µManager software [40]. Twofold binning resulted in 696 × 520 pixels images, with one pixel encompassing 2 × 2 µm. Trials were recorded at a rate of 4 Hz for 10 s. Odour stimulation began 2 s into recording, lasting 0.5 s.

Imaging data were analysed using the open-source platform FIJI [41] and Adobe Photoshop. For details of this process, see the extended methods in the electronic supplementary material. Briefly, data were corrected for bleaching and movement as adapted from other protocols [37]. Brains from different individuals were aligned using anatomical landmarks (electronic supplementary material, figure S4). An ROI overlapping the three target glomeruli identified with backfilling in the right AL was analysed (figure 3; electronic supplementary material, figure S3). The average response was measured by calculating the fluorescence change against the background ($\Delta F/F_0 = (F - F_0)/F_0$) and the peak $\Delta F/F_0$ after stimulus onset was determined. All frames were normalized to the highest fluorescence response value within each individual ($\Delta F/F_0$ (%)). Normalization allowed data pooling within races and statistical comparison between races. For statistical testing, we calculated a normalized difference index $(\Delta F/F_0\,3\,\mathrm{MB} - \Delta F/F_0\mathrm{BH})/(\Delta F/F_0\,3\,\mathrm{MB} + \Delta F/F_0\mathrm{BH})$, then used Welch's *t*-test (for unequal variances) to compare the races. To create final images for comparison, false colour-coded activity maps showing average fluorescence for each race were generated.

(f) Intracellular recordings and labelling

Animals were dissected to expose the antennal lobe as described above for optical imaging [37]. Intracellular sharp electrode recordings [42] to identify neurons that responded to the key volatiles took place at 60× magnification. Glass electrodes were created from borosilicate tubing (1.0 mm OD, 0.5 mm ID, World Precision Instruments, Sarasota, FL, USA) using a micropipette puller (P-1000, Sutter Instrument, Novato, CA, USA). Glass electrodes with resistance at least 80 MΩ in solution and over 100 MΩ in tissue were filled with 4% neurobiotin. The recording electrode was inserted randomly into the AL using a micromanipulator (Scientifica, Uckfield, UK). When neuronal contact was established, the antennae were stimulated and the analogue signal was amplified and filtered (Bramp Bridge Amplifier, npi Electronic GmbH, Tamm, Germany) and then synchronized with the external stimulation device via breakout box (INT-20MX Breakout Box Module, npi Electronic). Signals were monitored on an oscilloscope (TDS 1072B-EDU, Tektronics Inc., Beaverton, OR, USA). The resultant signal was digitized using a PCI card (6014, National Instruments, Austin, TX, USA) and captured using LabView SE 2013 (National Instruments).

If a neuron responded to BH not 3MB, or vice versa, and persisted for 5 min, we attempted to fill with neurobiotin using 1.4–2.8 nA of pulsed depolarizing current for 20 min. These flies were then brought through the staining procedure and imaged as for OSN backfilling, above.

Resulting *z*-stacks were compared to the AL reconstruction to determine where filled, ALNs which responded to 3MB or BH arborized. Verification involved several plug-ins in FIJI (rolling ball background subtraction [43,44], the Simple Neurite Tracer plug-in [45], see electronic supplementary material, figure S5, and image calculator). Population-level differences in neuronal responses to 3MB or BH were also generated (electronic supplementary material, figure S6) using the quickspike package [46]. For further details, see the extended methods in the electronic supplementary material.

3. Results

(a) Brain reconstruction

The structure of the *Rhagoletis* brain was very similar to that of *Drosophila melanogaster* [30], and thus we used standardized *D. melanogaster* nomenclature to designate regions [32]. We identified 47±2 glomeruli in *R. pomonella* ($n = 6$ reconstructions; figure 3*a–c*; electronic supplementary material, 3D PDFs S1 and S2), similar to *D. melanogaster* ($n = 54$) [30] and *Ceratitis capitata* ($n = 53$) [47].

(b) Input to the antennal lobe

Using our reconstruction of the *Rhagoletis* brain as a reference, we next performed single-sensillum electrophysiology combined with anterograde backfilling of the OSNs responsive to BH and 3MB to determine whether there was a difference in input to the AL between the host races [22]. We found that BH and 3MB responsive neurons innervated the same set of three glomeruli in apple ($n = 9$) and hawthorn ($n = 10$) flies, identified as the dorsal medial 1 (DM1), and ventral posterior 1 and 2 (VP1 and VP2) glomeruli (figure 3*d–f*; electronic supplementary material, figure S2). Note that while all three glomeruli were not labelled in every trial (electronic supplementary material, table S1), only these glomeruli were identified in targeted backfills. Thus, there appears to be no difference in the overall input to the antennal lobe by OSNs responding to BH or 3MB between the apple and hawthorn races. However, backfilling resulted in the indiscriminate labelling of both BH and 3MB responsive neurons as they are colocalized together within sensilla. Consequently, we were unable, with this method, to discern which volatile activated which glomerulus.

(c) Differential activity in the antennal lobe

To obtain an overview of neural activity in the AL in in the apple and hawthorn races of *R. pomonella* as they responded to key volatiles, we employed a bath-applied fluorescent dye commonly used for imaging non-transgenic species [37,38]. Thirteen of 150 animals responded to both BH and 3MB in the same preparation and were retained for analysis. We used solvent controls and a non-host odour (1-octen-3-ol) to standardize activity patterns. Then activity to BH versus 3MB was compared within the specific region of the antennal lobe (figure 4*a*; electronic supplementary material, figure S3) that contained the three glomeruli of interest (DM1, VP1 and VP2 identified via OSN backfilling). We employed this strategy to select an ROI because the thick neurilemma membrane [48] and bath-applied dye [49] prevented the localization of the individual glomeruli of interest, a known limitation for this technique.

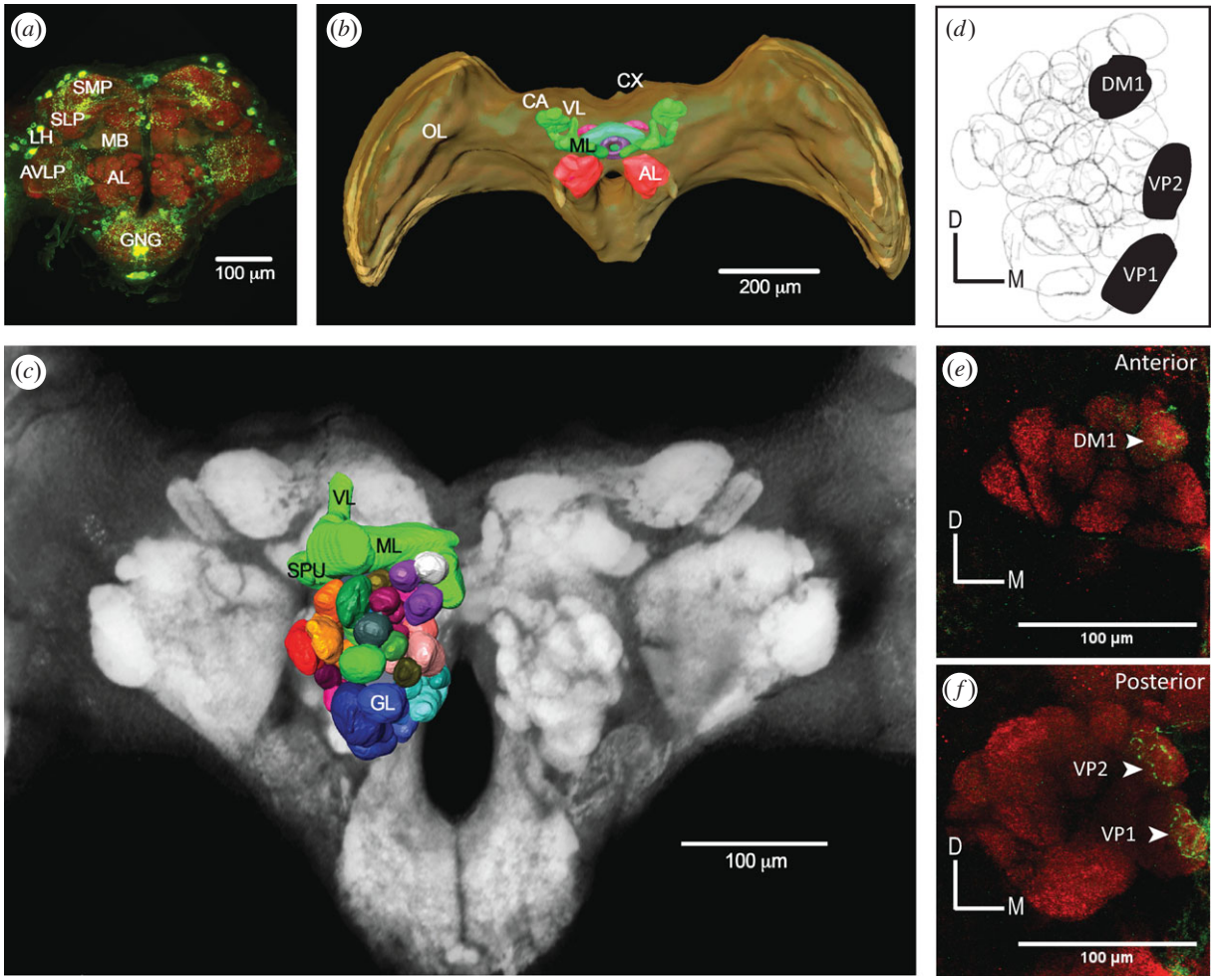

Figure 3. *Rhagoletis pomonella* brain anatomy and identification of glomeruli innervated by host volatile responsive OSNs. (*a*) Confocal Z-projection micrograph (75 µm depth) showing synapsin (red) and FMRFamide-like neuron immunoreactivity (green). Anatomical regions are identified: superior medial protocerebrum (SMP), superior lateral protocerebrum (SLP), lateral horn (LH), anterior venterolateral protocerebrum (AVLP), mushroom body (MB), antennal lobe (AL) and gnathal ganglia (GNG). (*b*) Three-dimensional reconstruction based on histological sections showing the optic lobe (OL), mushroom body calyx (CA), mushroom body ventral lobe (VL), mushroom body medial lobe (ML), central complex (CX) and AL. (*c*) Three-dimensional reconstruction of the AL and the MB indicating the spur (SPU) overlaid onto a Z-projection micrograph (300 µm depth). The glomeruli (GL) are labelled with different colours. (*d*) Three-dimensional reconstruction with glomeruli (DM1, VP1 and VP2) targeted by OSNs responsive to the volatiles BH and 3MB in black. (*e*,*f*) Confocal Z-projection of the AL following neurobiotin backfilling of OSNs (green), targeting glomerulus DM1 (*e*, arrowhead) and targeting glomeruli VP1 and VP2 (*f*, arrowheads). The synaptic marker nc82 (red) labels background neuropil. (Online version in colour.)

Images of apple ($n = 6$) and hawthorn ($n = 7$) fly ALs at peak response to BH and to 3MB (as in figure 4*b*) were aligned by choosing physical landmarks (electronic supplementary material, figure S4). These images were averaged to create representative activity maps (figure 4*c*,*d*), which showed that the hawthorn race displayed a greater response intensity to 3MB relative to BH (figure 4*e*), while the apple race showed the opposite pattern (figure 4*f*). To quantify the neural activity between the host races, we calculated a normalized difference index ($\Delta F/F_0$ 3MB $- \Delta F/F_0$ BH)/($\Delta F/F_0$ 3MB $+ \Delta F/F_0$ BH) for each individual, which was found to be significantly different between races (figure 4*g*). Such a change in activity indicates that apple and hawthorn race flies exhibit differential olfactory processing of host volatiles at the first olfactory synapse, the antennal lobe.

(d) Output from the antennal lobe

While optical imaging showed a significant difference in the neural processing of host odours between the races at the level of the antennal lobe, we were not able to identify changes in AL targeting of BH and 3MB responsive OSNs with peripheral backfilling. We thus used intracellular recording of ALNs coupled with backfilling to examine the output of information to higher brain centres [42]. To identify which glomeruli (DM1, VP1, VP2) are involved in processing BH versus 3MB, we recorded from ALNs that responded either to BH or 3MB, not both. We prepared 116 individuals, observing at least one neuronal contact in 78. In 50 animals, we attempted to fill neurons that had responded separately to either BH or 3MB. Of those, 13 were not labelled, 27 resulted in partial or non-target labelling, with the remaining 10 further analysed as ALNs with observed projections to higher processing centres (electronic supplementary material, figure S5).

ALNs in hawthorn flies responding to 3MB only and not BH arborized in the VP1 and VP2 glomeruli ($n = 3$), while in apple flies, ALNs responding to 3MB only arborized in the DM1 glomerulus ($n = 3$) (figure 5; electronic supplementary material, figure S6). Conversely, in hawthorn flies, ALNs responding to BH only and not 3MB arborized in DM1 ($n = 2$), while in apple flies, ALNs responding to BH only arborized in VP1 and VP2 ($n = 2$; figure 5; electronic supplementary material, figure S6). ALNs grouped glomeruli

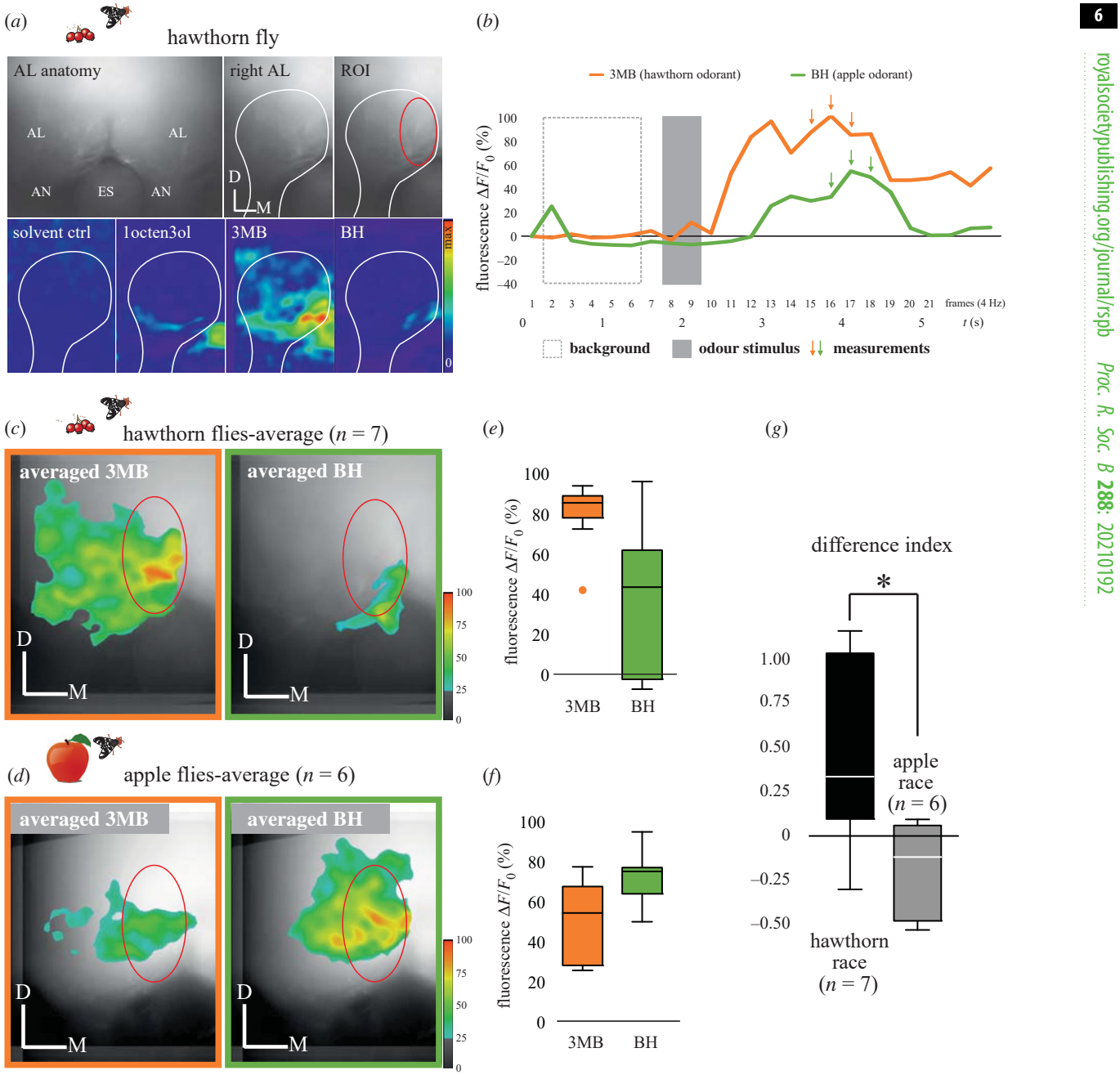

Figure 4. Differences in brain activity while responding to fruit volatiles. (*a*, top) Greyscale image of right antennal lobe (AL) with antennal nerve (AN) and oesophagus (ES), and with a small ROI encompassing the DM1, VP1 and VP2 glomeruli in red. (bottom) False colour-coded responses of an individual hawthorn fly to the solvent control, 1-octen-3-ol, 3MB and BH, normalized to the highest response within that animal. (*b*) Fluorescence change over time in response to 3MB (orange) and BH (green) in the same hawthorn fly as in *a*. (*c*) Anatomical greyscale images overlaid with averaged false colour-coded responses of hawthorn flies and (*d*), the same in apple flies, responding to 3MB (orange box) and BH (green box). (*e*) Boxplots summarizing hawthorn fly peak neural activity and (*f*) the same in apple flies. (*g*) Boxplot showing the normalized difference index for each race (significantly different, Welch's *t*-test, d.f. = 8, $t = 2.707$, $p = 0.0268$). (Online version in colour.)

VP1 and VP2 together consistently (in 4/5 total labelled neurons), and no identified neurons arborized all three glomeruli, suggesting that VP1 and VP2 specifically process one host odour, and DM1 the other.

We note that several of these ALNs responding to one of the two host odours also showed diffuse staining in non-target glomeruli or in other regions of the brain (e.g. the GNG), in addition to specific staining within DM1, VP1 and VP2. Therefore, as a conservative measure to avoid confirmation bias in observing glomerular arborization, we separately quantified the average fluorescence intensity within each glomerular region by calculating a normalized difference index (ΔF DM1/ΔF VP1 and VP2, electronic supplementary material, extended methods) to compare fluorescence within the three glomeruli. In hawthorn flies, BH responsive ALNs showed higher fluorescence intensity levels in the DM1 glomerulus, while 3MB responsive ALNs showed higher in VP1 and VP2 (figure 5*c*). By contrast, for apple flies, BH responsive ALNs showed higher fluorescence intensity in the VP1 and VP2 glomeruli, while 3MB responsive ALNs showed higher in DM1 (figure 5*f*). Thus, the main host volatiles are processed in different regions of the AL that are reversed between the apple and hawthorn host races. Coupled with the difference in neural activity shown by optical

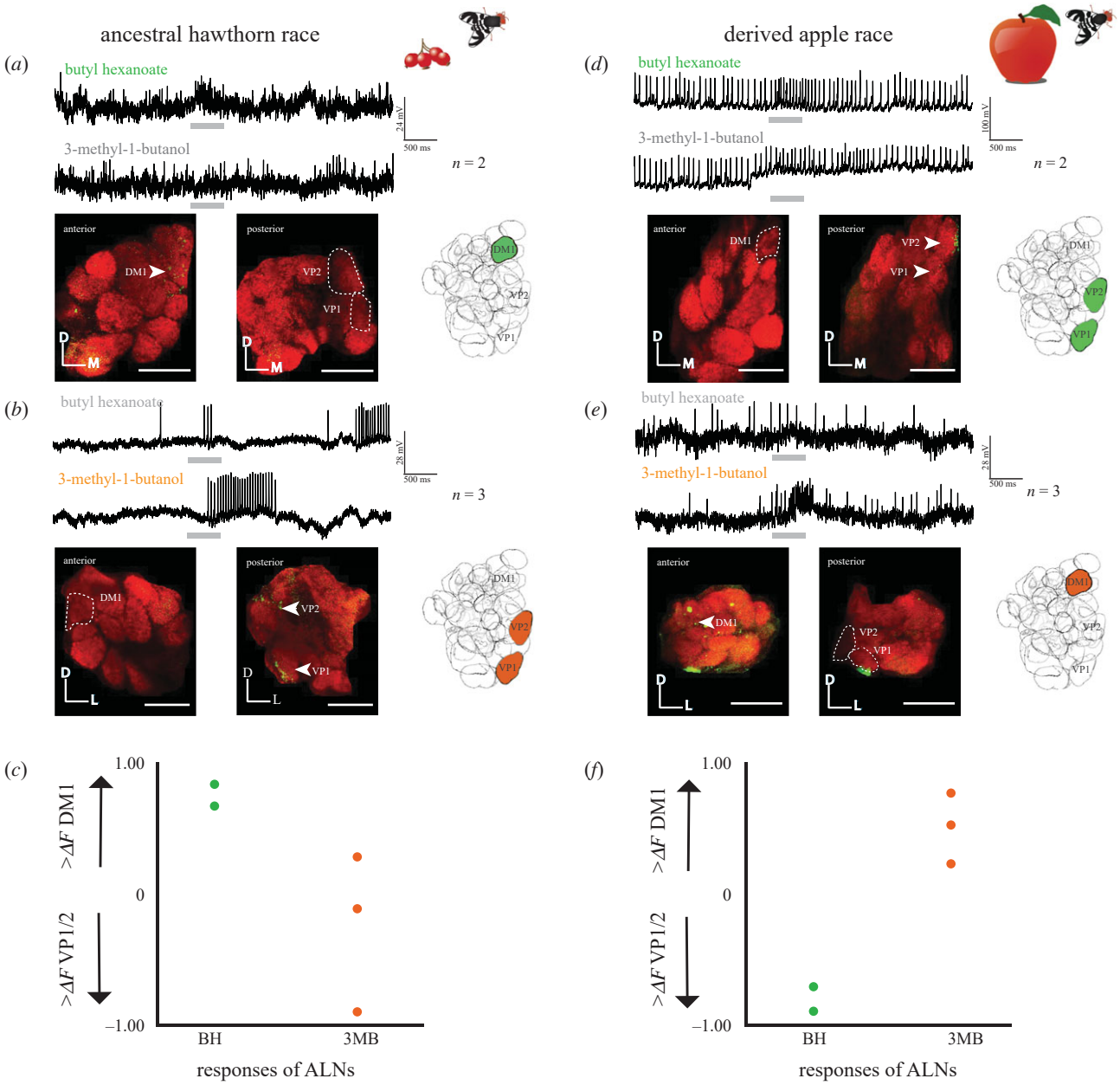

Figure 5. Targeting of ALNs responding to key volatiles. (*a,b,d,e*) (top) Paired 5 s intracellular recording traces for ALNs responding to BH (top) or 3MB (bottom). A volatile prompted a response if the volatile's name is in colour. Grey lines below indicate the 0.5 s stimulus. (bottom) Maximum confocal Z-projections (10–20 µm anterior, left, and 10–20 µm posterior, right) of labelled ALNs corresponding to the spike recordings, using neurobiotin (green), counter-labelled with synaptic marker nc82 (red). Scale bars, 50 µm. ALNs targeting DM1, VP1 or VP2 glomeruli indicated with white arrowheads; glomeruli lacking arbourization are outlined by dotted lines. Insets for each panel summarize glomeruli targeted by ALNs responding to BH (green; *a,d*) or 3MB (orange; *b,e*). (*c,f*) Scatter plots showing the normalized difference index of average fluorescence intensity within the three target glomeruli (ΔF DM1 − ΔF VP1and2)/(ΔF DM1 + ΔF VP1and2) for confocal Z-projections of the ALNs responding to either BH or 3MB in each race. (Online version in colour.)

imaging, these results indicate that the principal attractive volatile for both host races (BH for apple and 3MB for hawthorn flies) is associated with VP1 and/or VP2 glomeruli, while the antagonistic volatile is associated with DM1.

4. Discussion

Our results imply that a reversal in processing of two critical volatiles (BH and 3MB) differentially affecting host fruit choice in apple and hawthorn race flies occurs at the first olfactory synapse in the brain, the antennal lobe. Earlier studies found that OSNs responsive to BH or 3MB are colocalized in the same sensilla in the antenna of both apple and hawthorn flies. Similar features in olfactory processing, including colocalization of key OSNs on the antennae and reversals in odour-evoked activity at the level of the antennal lobe, are associated with changes in sex pheromone systems in moths [23–26]. These similarities suggest evolutionary convergence in the neural mechanisms associated with behavioural divergence in mate and habitat choice, generating prezygotic ecological reproductive isolation. In *R. pomonella*, host fruit volatiles are the critical first signal and are sufficient for finding mates on host plants without the need for long distance sex pheromones.

The known history of the recent shift of *R. pomonella* to apple and the partial but not complete isolation of the apple from the hawthorn race are important, because this indicates the reversal in BH and 3MB processing is

contributing to active divergence rather than evolving after speciation is complete. Thus, changes in the antennal lobe can be directly tied to initiating rapid population divergence. Future work is still needed to identify the genetic cause and developmental basis for the reversal in processing of BH and 3MB. Leading candidates include differential expression of receptor proteins on the OSNs (figure 2*a*), as appears to be the case in the sex pheromone systems of heliothine moths [25,26] and/or developmental changes in neuronal targeting of OSNs from the antennae glomeruli in the antennal lobe glomeruli (input to figure 2*b*), as in European corn borer moth [23,24]. It remains possible that more complex changes in processing within the antennal lobe itself, prior to the ALN level output (figure 2*b*,*c*), contribute to the observed differences via interneurons, neuromodulators or centrifugal input from higher processing centres. Furthermore, the *Rhagoletis* genus contains many specialist taxa attracted to the volatiles of specific hosts, providing the opportunity to expand analysis across the speciation continuum from partially isolated races, like apple and hawthorn flies, to fully differentiated species.

In conclusion, we have identified a reversal in processing at the first olfactory synapse, the antennal lobe of the brain, associated with odours key for differential host choice behaviour and thus involved in the rapid ecological divergence of *R. pomonella*. Coupling of habitat and mate choice is common for host specialist phytophagous insects and their parasites [5], and often involves olfactory cues. The reversal in olfactory processing in *Rhagoletis* may be a common theme contributing to the reproductive isolation of many phytophagous insects. Host-seeking behaviour connecting adult reproduction directly with larval feeding and survival, here shown to involve relatively simple changes in sensory processing, could be part of the explanation for the great diversity of insect specialists noted by Walsh and Darwin. Indeed, such changes in sensory processing may be a broad mechanism for generating biodiversity across sensory modalities and for many groups in addition to phytophagous insects, constituting a major factor contributing to the origin of species.

Data accessibility. Raw data files supporting this manuscript are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.bnzs7h49 [50].

Authors' contributions. C.T., S.B.O. and J.L.F. conceived the study; C.T. and S.B.O. designed experiments; C.T., H.K. and D.K. conducted experiments; C.T., H.K., D.K., M.S., A.S., J.R., J.L.F. and S.B.O. analysed data; C.T., S.B.O. and J.L.F. wrote the manuscript; all authors revised and approved the manuscript.

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