Insect outbreaks produce distinctive carbon isotope signatures in defensive resins and fossiliferous ambers

Ryan C. McKellar1,* , Alexander P. Wolfe1, Karlis Muchlenbachs1, Ralf Tappert1, Michael S. Engel2, Tao Cheng3 and G. Arturo Sánchez-Azofeifa1

1Department of Earth and Atmospheric Sciences, University of Alberta, 1-26 Earth Sciences Building, Edmonton, Alberta, Canada T6G 2E3
2Division of Entomology, Natural History Museum and Department of Ecology and Evolutionary Biology, University of Kansas, 1501 Crestline Drive, Suite 140, Lawrence, KS 66049-2811, USA
3Department of Land, Air and Water Resources, University of California, One Shields Avenue, Davis, CA 95616, USA

Despite centuries of research addressing amber and its various inclusions, relatively little is known about the specific events having stimulated the production of geologically relevant volumes of plant resin, ultimately yielding amber deposits. Although numerous hypotheses have invoked the role of insects, to date these have proven difficult to test. Here, we use the current mountain pine beetle outbreak in western Canada as an analogy for the effects of infestation on the stable isotopic composition of carbon in resins. We show that infestation results in a rapid (approx. 1 year) $^{13}$C enrichment of fresh lodgepole pine resins, in a pattern directly comparable with that observed in resins collected from uninfested trees subjected to water stress. Furthermore, resin isotopic values are shown to track both the progression of infestation and instances of recovery. These findings can be extended to fossil resins, including Miocene amber from the Dominican Republic and Late Cretaceous New Jersey amber, revealing similar carbon-isotopic patterns between visually clean ambers and those associated with the attack of wood-boring insects. Plant exudate $\delta^{13}$C values constitute a sensitive monitor of ecological stress in both modern and ancient forest ecosystems, and provide considerable insight concerning the genesis of amber in the geological record.

Keywords: mountain pine beetle; Dominican amber; New Jersey amber; stable isotopes

1. INTRODUCTION

The stable isotopic composition of plant carbon has the capacity to record physiological stress, such as that induced by insect attack, because $^{13}$C discrimination is reduced under impaired conditions [1]. Although carbon stable isotopes have been considered from a range of plant tissues (e.g. [2–5]), relatively little attention has been paid to exuded secondary metabolites [6–10], despite their excellent potential for preservation in the fossil record as amber [11,12]. Resin retains its original stable isotopic composition throughout maturation with a high degree of fidelity [7,10], making analogies between modern resin and fossil amber tenable. Within a framework of actualistic palaeontology, we examined the role of wood-boring insects as a potential cause for amber production by investigating the isotopic response of modern resins generated in direct response to a major insect outbreak event.

Mountain pine beetle (Dendroctonus ponderosae, Coleoptera: Curculionidae: Scolytinae; hereafter MPB) is currently devastating western North American forests, with pronounced economic, ecological and biogeochemical consequences [13]. Climate change (specifically, warmer winter temperatures) has allowed MPB to infest extensive tracts of lodgepole pine (Pinus contorta var. latifolia; figure 1) that are already weakened by multiple years of drought. MPB larvae over-winter in galleries excavated in the host tree’s cambium, where they feed on blue stain fungus (Grommannia clavigera, Ascomycota). Adults emerge in late summer, disperse and form mass attacks guided by combinations of pheromones and volatile resin constituents released during pioneer attacks [14]. This strategy frequently overcomes the constitutive (preformed) oleoresin defense system of the host tree (known as pitch-out), while MPB boring introduces fungi that act as a food source as galleries develop. Once galleries and the spread of fungi have girdled the tree, there is further impairment of both induced (secondary) resin production and the mechanics of water circulation in the phloem [15]. Foliage of infested trees first dulls then yellows and reddens over 1–2 years following a successful MPB attack [13]. By the time foliage of infested trees becomes visibly impacted on a large scale, MPB has often dispersed to new hosts.

The example of MPB provides a powerful analogy to explore $\delta^{13}$C patterns in geologically relevant volumes of plant resins and test hypotheses concerning the origins...
of amber deposits. In the course of developing a database of resin and amber stable isotopic compositions for the major world deposits, sufficient material was acquired and analysed to examine in detail two major deposits that are putatively associated with insect damage: Dominican and New Jersey (NJ) amber.

Abundant deposits of fossiliferous Miocene amber from the Santiago region of the Dominican Republic have been attributed with confidence to the leguminous tree genus *Hymenaea* [17], for which modern taxa produce large volumes of resin in response to injury [11,17]. Dominican amber is considered to have formed as a result of catastrophic events in an established moist tropical forest [18]. Hurricane damage has been invoked as the most likely factor leading to the massive production of resin within this forest [19,20], ultimately producing the amber deposit. The large number of bark beetles preserved as inclusions within the amber [18,21] suggests an alternative cause, and provides a unique opportunity for comparison with modern resins produced as a direct result of infestation by scolytine beetles like MPB.

Late Cretaceous (Turonian) amber from NJ has been associated with both forest fires and the activities of wood-boring beetles with associated pathogenic fungi [22]. Although amber from this deposit is chemically homogeneous and potentially originates from a single source conifer taxon, three distinct visual categories emerge from the census of thousands of specimens examined [22]. Approximately 70 per cent of specimens are turbid, and contain the majority of insect inclusions as well as copious wood particulate material directly comparable with that produced by insect boring [22]. Most remaining specimens comprise small droplets that are optically transparent, but a third category includes rare specimens of opaque amber having a frothed appearance from microscopic gas inclusions associated with burning [22]. Only the opaque amber can be related directly to fire events given that, beyond its distinctive bubble-rich appearance, inclusions of fusainized wood are also found within. The presence of fusainized plant remains within the surrounding clay has led to the suggestion that the entire amber deposit resulted from forest fires [22], but it remains unclear whether this sedimentological association is the result of taphonomic processes [23,24].

2. MATERIAL AND METHODS

(a) Samples

We sampled lodgepole pine resins in an experimental forest plot near Grande Prairie, Alberta, Canada (55°05′13″ N, 118°12′54″ W), in both 2008 and 2009. Lodgepole pine resins were collected from infested and uninfested trees in close proximity in order to rule out the effects of microhabitat variability. Resin was collected from fresh flows on the lower trunk or from freshly trimmed branches. For NJ and Dominican amber, isotopic measurements were made from marginal cuttings of barren and insect-bearing specimens, avoiding bubbles and all particulate inclusions.

(b) Methods

Established methods were employed for carbon stable isotopic measurements and amber palaeoentomological investigations [8,25]. All isotopic data are reported in delta notation ($\delta^{13}C$) relative to VPDB (Vienna PeeDee Belemnite) with a precision of $\pm 0.1$ per mille. Modern resins were first heated (50°C) for 12 h under vacuum to remove the most volatile resin components and environmental water. Supplementary experiments with modern and subfossil *Hymenaea* resin showed no measurable fractionation associated with heating. All statistical differences between populations of resin and amber $\delta^{13}C$ values were assessed using Welch two-sided $t$-tests with 95 per cent confidence intervals. Mean values for each sample population are reported $\pm 1$ standard deviation. All results and sample descriptions are available online as electronic supplementary material (table S1).

3. RESULTS AND DISCUSSION

(a) Mountain pine beetle attack and resin carbon isotopes

During the 2008 MPB outbreak expansion, we observed $\delta^{13}C$ enrichment in resins from trees in the earliest stages of infestation (known as green attack), compared with adjacent healthy control trees (figure 2). The difference
### δ¹³C (VPDB) %

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<tr>
<td>modern Pinus contorta resins</td>
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<td>MPB 2008 control trees</td>
<td>–28.2 ± 0.68‰</td>
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<td>MPB 2008 green attack</td>
<td>–27.0 ± 0.91‰</td>
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<td>MPB 2009 control, surviving trees</td>
<td>–28.0 ± 0.65‰</td>
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<td>MPB 2009 infested, surviving trees</td>
<td>–28.2 ± 0.80‰</td>
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<td>MPB 2009 yellowing</td>
<td>–26.4 ± 0.22‰</td>
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<td>MPB 2009 red attack</td>
<td>–23.2%</td>
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<td>root-bound nursery trees</td>
<td>–26.9 ± 0.81‰</td>
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<tr>
<td>modern Hymenaea courbaril resin</td>
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<td>sub-recent Colombian copal</td>
<td>–28.3 ± 0.71‰</td>
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<td>barren Dominican amber</td>
<td>–27.5 ± 0.55‰</td>
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<td>Dominican amber, insect inclusions</td>
<td>–24.9 ± 1.54‰</td>
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<td>Dominican amber, boring-beetle inclusions</td>
<td>–23.6 ± 1.25‰</td>
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<td>New Jersey Cretaceous amber</td>
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<tr>
<td>barren</td>
<td>–22.3 ± 1.19‰</td>
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<td>turbid</td>
<td>–21.9 ± 0.98‰</td>
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<td>opaque</td>
<td>–20.6 ± 0.37‰</td>
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**Figure 2.** δ¹³C values of modern resins and amber analogues. The mean and standard deviation of each sample population are shown beneath the data. Colours correspond to inferred levels of tree ecophysiological stress, based on consensus of the extent of observed insect damage and attendant mean exudate δ¹³C value within each population, ranging from healthy (dark green) to near death (red).

between 2008 newly infested green attack trees (mean δ¹³C = –27.0 ± 0.91‰) and uninfested control trees (mean δ¹³C = –28.2 ± 0.68‰) is highly significant (p = 0.018), with resins from infested trees consistently enriched relative to neighbouring healthy trees subjected to identical microclimatic conditions. In one tree where superimposed resin flows could be dissected sequentially to capture conditions prior to and during MPB attack, resin δ¹³C values shifted from –28.2 (pre-2008) to –26.7 per mille (2008). Freshly produced resins from uninfested lodgepole pine trees that had been subjected to water stress for four months since excavation and bagging of the root mass yielded mean δ¹³C = –26.9 ± 0.81 per mille. A similar isotopic enrichment to that induced by MPB attack can thus be induced rapidly by water stress alone, supporting the notion that phloem interruption is the most likely mechanism mediating isotopic enrichment in both cases.

Additional lodgepole pine resins from the Grande Prairie test plot were collected in 2009, following a relatively harsh winter and late spring that slowed MPB expansion. Surviving infested trees produced new defensive resins that registered a return to pre-infestation values (mean δ¹³C = –28.2 ± 0.80‰), indistinguishable from either 2008 or 2009 healthy control trees (figure 2). Both surviving populations are in stark contrast with trees developing visible yellow foliage (mean δ¹³C = –26.4 ± 0.22‰). By far the most enriched sample was from a tree entering red attack (δ¹³C = –23.2‰), which provides an important end-member because few red attack specimens retain the capacity to exude new resin. These analyses demonstrate that the trajectory of MPB outbreak in lodgepole pine resin is isotopically discernible in real time: MPB attack induced positive δ¹³C excursions averaging 1.1 per mille that intensify if the tree eventually succumbs, but are rapidly reversed in cases of rebound.

(b)** Dominican amber and Hymenaea resins**

Modern *Hymenaea courbaril* resin (mean δ¹³C = –28.3 ± 0.71‰) and subfossil *Hymenaea* copal (mean δ¹³C = –27.5 ± 0.55‰) constrain the isotopic composition of resins derived from wounding of otherwise healthy trees. Values from Dominican amber are enriched relative to these baseline values (figure 2), and progress from specimens barren of insects (mean δ¹³C = –26.0 ± 1.01‰) to those with inclusions of generalist insects unrelated to infestation (mean δ¹³C = –24.9 ± 1.54‰) and finally to amber containing obligate tree-boring beetles of the subfamilies Scolytinae and Platypodinae (Curculionidae, figure 3b; mean δ¹³C = –23.6 ± 1.25‰). The latter population’s isotopic composition is significantly different (p = 0.011) from that of other Dominican amber specimens and *Hymenaea* resins.

When compared with the MPB dataset, the distribution of δ¹³C values observed in Dominican amber strongly suggests that many of the pieces containing boring beetles were produced at times of increased tree stress, probably as a direct result of insect attacks. In a survey of 2924 Dominican amber arthropod inclusions, 115 platypodine beetles were observed [18], while others have noted that scolytines comprise approximately 7 per cent of insect inclusions within the deposit [26]. Wood-boring beetles are thus a prominent group within the entombed fauna, and their activities may have contributed meaningfully to amber production. For example, remaining barren Dominican amber specimens present intermediate δ¹³C values relative to *Hymenaea* resins produced as a result of mechanical damage and those containing fossil insects (figure 2). This suggests that mechanical injury alone was insufficient to produce the isotopic compositions observed in the latter population.

(c)** New Jersey amber**

All three visual classes of NJ amber (figure 3c) were analysed isotopically, revealing a progression of δ¹³C
enrichment between fragments of transparent (mean $\delta^{13}C = -22.3 \pm 1.19\%$), turbid (mean $\delta^{13}C = -21.9 \pm 0.98\%$) and opaque (mean $\delta^{13}C = -20.6 \pm 0.37\%$) amber. This distribution indicates that many amber specimens containing insect inclusions and plant debris were formed from resins produced under conditions of pronounced ecophysiological stress, thus supporting the association between insect-mediated damage and defensive resin production. The further enrichment observed in opaque NJ amber is consistent with the loss of isotopically depleted volatile moieties during fire events, as implied independently by the record of bubble and fusain inclusions.

Although the direction and amplitude of these isotopic shifts are consistent with the MPB results, we note that the baseline $\delta^{13}C$ of NJ amber is consistently enriched (by approx. 5%) relative to modern lodgepole pine resins. We suggest that combinations of source tree metabolism, fundamental differences in the Cretaceous carbon cycle [27] and slight degrees of long-term resin diagenesis explain these differences, but that the original fingerprint of tree physiological stress is nonetheless recorded by amber isotopic compositions. Despite these potential influences, the distribution of $\delta^{13}C$ values retains a clear pattern within the deposit, which is directly comparable with the pattern produced by MPB infestation.

One limitation of invoking wood-boring beetles in the genesis of NJ amber is their relative scarcity as inclusions within the amber. In a survey of 1032 NJ amber arthropod inclusions [22], 37 identifiable beetles were recovered, two of which belonged to live-wood-boring groups (Cupedoida). Scolytinae and Platypodinae are known from Early Cretaceous Burmese amber, and Scolytinae are also present in Early Cretaceous Lebanese amber [26], but both groups have yet to be recovered from NJ amber. While the presence of obligate beetle parasitoids (such as wasps of the families Megalyridae, Orussidae and Scolebythidae [22,28]) provides indirect evidence for wood-boring beetles, at present, the abundance of wood particulates within turbid amber provides the strongest independent evidence for wood-boring (figure 3d). These indirect inferences are now augmented convincingly by the results of our isotopic analyses.

4. CONCLUSIONS
The direction and magnitude of resin $\delta^{13}C$ values from Dominican and NJ amber mirrors that expressed in MPB-infested pine forests (figure 2). In each instance, a strong case can be made for the association of insects to physiological stress in host trees, which in turn has the potential to become recorded in the $\delta^{13}C$ of resins synthesized shortly after attack. As a corollary, not all ambers preserving inclusions of wood-boring insects have strongly enriched isotopic values, as expected given opportunities for recovery that may equally be registered by $\delta^{13}C$. For example, although a 1.3 per mille increase in mean $\delta^{13}C$ values was observed in Dominican amber samples containing boring beetles, there is substantial overlap with specimens lacking direct evidence of insect attack. We envisage that much of the isotopic variability documented originates in water stress mediated by insects and their fungal symbionts, which affects both the quantity and quality of defensive resins. Water stress results in the progressive enrichment of the carbon-isotopic composition of photosynthetic products by reducing stomatal conductance, and hence the ability to discriminate against $^{13}C$ [1]. We have shown here that this isotopic effect is propagated to secondary metabolites. Boring insect attacks induce the same general pattern of isotopic
enrichment produced by drought alone, because the attacks interfere with water transport in host trees. As a corollary, pre-conditioning by drought clearly has been a factor in the magnitude and severity of current MPB outbreaks [13].

In modern and ancient ecosystems, δ13C values from tree resins constitute a novel early indicator of insect outbreak events. This tool can be applied to ambers as old as the Mesozoic, and is expressed in both conifer and angiosperm resins. More intensive work is required to disambiguate the isotopic effects specific to insects and plant water stress, but our work addressing MBP suggests that resin composition responds faster than foliage colour, and hence can diagnose the severity of an outbreak more rapidly than satellite remote sensing, with implications for management. The analogies with both Cretaceous and Miocene ambers provide new lines of evidence supporting the active participation of insects in the original production of resins now preserved as amber, and correlate nicely with the diversity of wood-boring insects and their associates recovered from these deposits. Carbon stable isotopic ratios in fossil resins, together with detailed palaeoentomological consideration of associated inclusions, can be exploited to better understand this important dimension of plant–insect coevolution.

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