

# Ambrosia Beetle (Coleoptera: Curculionidae) Communities and Frass Production in ‘Ōhi‘a (Myrtales: Myrtaceae) Infected With *Ceratocystis* (Microascales: Ceratocystidaceae) Fungi Responsible for Rapid ‘Ōhi‘a Death

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## Abstract

Rapid ‘Ōhi‘a Death (ROD) is a deadly disease that is threatening the native Hawaiian keystone tree species, ‘Ōhi‘a lehua (*Metrosideros polymorpha* Gaudich). Ambrosia beetles (Curculionidae: Scolytinae) and their frass are hypothesized to play a major role in the spread of ROD, although their ecological niches and frass production within trees and across the landscape are not well understood. We characterized the beetle communities and associated frass production from bolts (tree stem sections) representative of entire individual ‘Ōhi‘a trees from multiple locations across Hawai‘i Island by rearing beetles and testing their frass for viable ROD-causing fungi. Additionally, we estimated frass production for three beetle species by weighing their frass over time. We found that *Xyleborinus saxesenii* (Ratzburg), *Xyleborus affinis* Eichhoff, *Xyleborus ferrugineus* (Fabricius), *Xyleborus perforans* (Wollaston), and *Xyleborus simillimus* Perkins were commonly found on ROD-infected ‘Ōhi‘a and each produced frass containing viable *Ceratocystis* propagules. The Hawai‘i Island endemic beetle and the only native ambrosia beetle associated with ‘Ōhi‘a, *X. simillimus*, was limited to high elevations and appeared to utilize similar tree heights or niche dimensions as the invasive *X. ferrugineus*. Viable *Ceratocystis* propagules expelled in frass were found throughout entire tree bole sections as high as 13 m. Additionally, we found that *X. ferrugineus* produced over 4x more frass than *X. simillimus*. Our results indicate the ambrosia beetle community and their frass play an important role in the ROD pathosystem. This information may help with the development and implementation of management strategies to control the spread of the disease.

**Key words:** Scolytinae, *Metrosideros*, frass, Rapid Ohia Death, *Ceratocystis*

‘Ōhi‘a lehua (*Metrosideros polymorpha* Gaudich) is an invaluable tree to both the Hawaiian landscape and culture (Mueller-Dombois et al. 2013). The tree is the dominant woody species in many ecological zones from lava fields to wet forest and occurs at all altitudinal ranges from sea level to treeline (Mueller-Dombois 1992, Cordell et al. 1998, Mueller-Dombois et al. 2013). On Hawai‘i Island alone, ‘Ōhi‘a covers approximately 250,000 ha (Gon et al. 2006). Much of the endemic biodiversity of Hawai‘i evolved with and rely heavily on ‘Ōhi‘a for food and shelter such as the Hawaiian honeycreepers (Aves: Drepanidini) and at least 500 different endemic arthropod species (Gruner 2004). Because of the commonality and usefulness of the wood, leaves, and flowers, ‘Ōhi‘a has been

incorporated into many aspects of the Hawaiian culture, from plant lore to woodworking, and has ultimately become a form of identity for some native Hawaiians (Abbott 1992, Mueller-Dombois et al. 2013).

Hawaiian ecosystems are particularly vulnerable to invasive species threats having evolved in the absence of many predators and diseases that are common in continental areas (Vorsino et al. 2014). In 2010, a new, devastating mortality event characterized by the rapid browning of leaves and defoliation of ‘Ōhi‘a trees was reported in the Puna District of Hawai‘i Island and termed Rapid ‘Ōhi‘a Death (ROD) (Keith et al. 2015). ROD was later determined to be caused by two novel fungal species of *Ceratocystis*: *C. lukuohia* I. Barnes,

T.C. Harrin. & L.M. Keith and *C. huliobia* I. Barnes, T.C. Harrin. & L.M. Keith (Barnes et al. 2018). *Ceratocystis lukuohia* is more virulent and common than *C. huliobia*, although both pathogens are fatal to ‘ōhi‘a and can sometimes co-infect the same tree (Barnes et al. 2018, Fortini et al. 2019, Hughes et al. 2020). ROD has killed at least one million trees on Hawai‘i Island (ROD Working Group, written communication, 2020) and the potential threat to native ecosystems has been compared to chestnut blight and Dutch elm diseases in North America (Mortenson et al. 2016). Although not yet fully understood, the dissemination of the pathogens from infected trees is hypothesized to be at least partially facilitated by ambrosia beetles (Curculionidae: Scolytinae) and their frass or boring dust (i.e., wood particles, feces, and beetle parts; Fig. 1A) (Heller and Keith 2018, Roy et al. 2019).

Ambrosia beetles can be highly invasive, many of which are associated with forest diseases (Hulcr and Dunn 2011, Rabaglia et al. 2019). Scolytid beetles of the tribe Xyleborini are particularly successful invaders of new ecosystems because of their wide range of host trees (Hulcr et al. 2007) and their parthenogenetic reproductive behavior (Kirkendall 1993) where a single unfertilized female can establish a population (Hulcr and Dunn 2011). Although rare, ambrosia beetles can directly transmit pathogens. For example, in the extreme case of *Xyleborus glabratus* and laurel wilt disease, a single founding female established a population that has killed over 320 million trees in the southeastern United States (Hughes et al. 2017). The potential for indirect movement of pathogens embedded in frass that is produced and expelled during gallery excavation is not well understood (Fig. 1B). Dislodged frass particles containing pathogens can be transported by soil, wind, and/or water, and ultimately inoculate an otherwise healthy tree by entering through a wound (Harrington 2013).

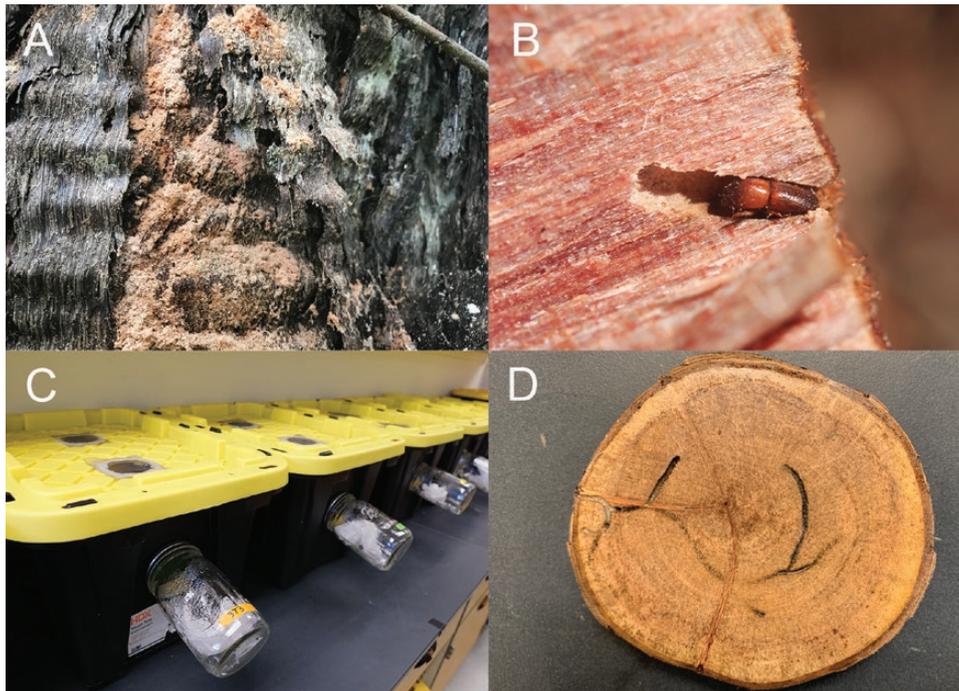
Several *Ceratocystis* diseases have been linked to ambrosia beetle frass including *C. platani* of plane trees (Ocasio-Morales et al.

2007), *C. cacaofunesta* of cacao trees (Itton 1961), and *C. fimbriata* of mango (Souza et al. 2013). Frass from *Ceratocystis*-infected trees can contain highly virulent conidia, hyphal fragments, and long-lived aleuroconidia spore types (Harrington 2009). In the Puna District of Hawai‘i Island, frass produced by both *Xyleborus ferrugineus* (Fabricius) and *Xyleborus perforans* (Wollaston) that was collected from the basal 1.5 m of ‘ōhi‘a trees was found to contain viable *Ceratocystis* propagules (Roy et al. 2019). However, it is unknown whether other ambrosia beetle species may be associated with ROD infections on Hawai‘i Island, if these beetles occupy specific positions in ROD-infected ‘ōhi‘a trees, and whether they are capable of producing ROD-infected frass. Understanding the height at which viable inoculum can be released into the environment in the form of frass and the variability in frass production from different species of beetles could improve the development of effective ROD management strategies. Currently, ROD managers are exploring options of tree felling and tarping as well as the use of insecticides (Keith et al. 2020).

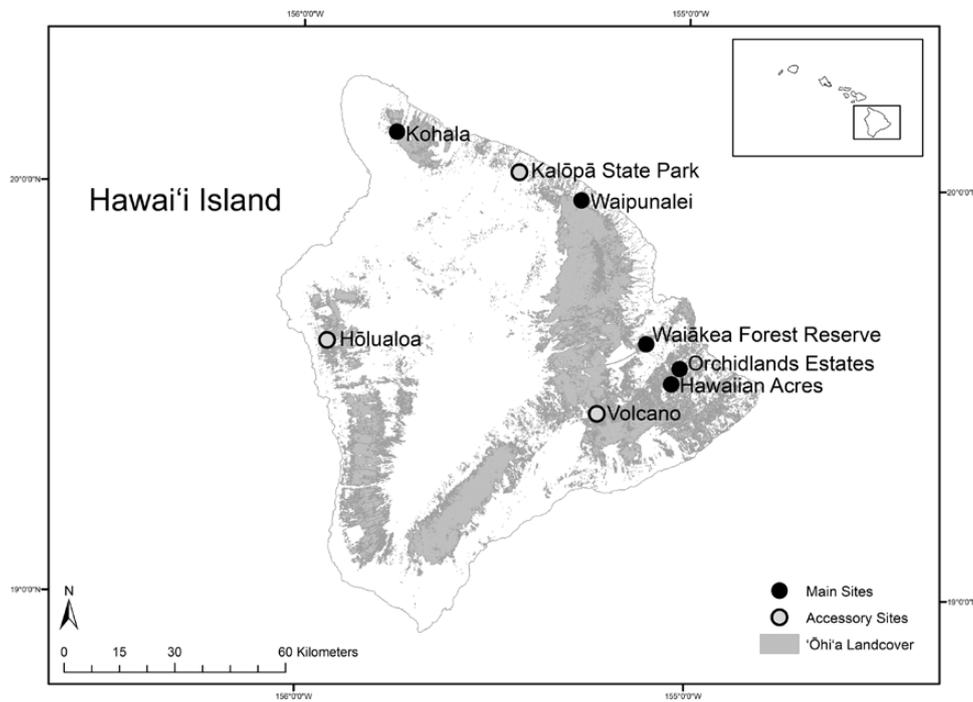
In this study, we collected ambrosia beetles and their frass from ‘ōhi‘a bolts (tree stem sections) representing entire tree boles from multiple locations on Hawai‘i Island. Our objectives were to 1) characterize the abundance, tree height niche dimension (position along the bole of the tree occupied by the beetles), and elevational distribution of ambrosia beetles associated with ROD-infected ‘ōhi‘a, 2) identify ambrosia beetle species that produce frass containing viable *Ceratocystis* propagules and the height at which this contaminated frass is produced, and 3) quantify the amount of frass produced by different ambrosia beetle species.

## Materials and Methods

Bolts representing the entire bole length of trees were obtained during August 2018–March 2019 at five locations on Hawai‘i Island.



**Fig. 1.** Examples of ambrosia beetles and their frass associated with ROD. (A) Ambrosia beetle frass accumulating on the outer bark of an ‘ōhi‘a tree, (B) *Xyleborus ferrugineus* excavating a gallery in ROD-infected ‘ōhi‘a, (C) emergence chambers for beetle rearing, and (D) ambrosia beetle gallery extending throughout the surface area of a *Ceratocystis*-infected ‘ōhi‘a cookie. Photo (B) by J.B. Friday, University of Hawai‘i at Mānoa.



**Fig. 2.** Locations of the five main study sites and three accessory sites where ROD-infected 'ōhi'a were collected across Hawai'i Island. Shaded gray areas represent 'ōhi'a forest landcover. Inset: Hawaiian Islands depicting the location of Hawai'i Island. 'ōhi'a landcover from Jacobi et al. (2017).

Sites included ROD-infected 'ōhi'a-dominant forests at Hawaiian Acres, North Kohala, Orchidlands Estates, Waipunalei, and Waiākea Forest Reserve, referred to as the 'main' study sites (Fig. 2). Trees were selected based on observations of wilt, beetle infestation, and the presence of *C. lukuobia* and/or *C. huliobia* DNA. *Ceratocystis* DNA was confirmed in each tree using the extraction and quantitative polymerase chain reaction (qPCR) methods described in Heller and Keith (2018). *Ceratocystis* DNA positive trees were felled, and five 50-cm bolts evenly spaced along the bole and proportional to the total bole length were measured and cut. Each bolt was then cut into two approximately equal lengths for ease of transportation. At the Hawaiian Acres site, one entire tree was cut into nineteen 50-cm bolts. To supplement the study, bolts from 'accessory' sites at Kalōpā State Park, Volcano, and Hōlualoa that did not represent the entire bole length were collected for a total of eight study sites (Fig. 2).

In the field, all bolts were measured and labeled with flagging tape. A sterilized draw knife was used to remove the outer layer of moss and bark to prevent the spread of the little fire ant (*Wasmannia auropunctata*), an aggressive invasive species that is spreading in Hawai'i (Lee et al. 2015). Bolts were then transported to the lab where the cut ends were sealed by brushing on melted Gulf Wax (Royal Oak Sales, Roswell, GA) paraffin and then placed in individual emergence chambers. The design of emergence chambers was based on modifications of Carrillo et al. (2012). Chambers were crafted from 12-, 17-, and 27-gal. (45-, 64-, 102-L) Tough Storage Totes, HDX (The Home Depot, Atlanta, GA) with screened holes for ventilation on the lids and connected by a 3-in (76-mm) diameter hole to a 1-quart (0.946-L) regular mouth Ball (Ball Corporation, Broomfield, CO) mason jar. Mason jars contained moistened paper towels that were replaced as needed (i.e., dried out, molding) (Fig. 1C). Boring Dust Traps (BDT), designed to collect frass and emerging beetles (Roy et al. 2019), were attached to the entrances of frass-producing beetle galleries and ranged from 0 to 16 BDT per bolt section ( $N = 178$ ). Emerging beetles were collected from both the mason jars and BDT at least

once per week and identified to species using Samuelson (1981) and Wood (1982). Voucher specimens were deposited at the University of Hawai'i at Mānoa Insect Museum, Honolulu, Hawai'i (accession numbers BS 20-00000001-00000115). Bolts were monitored for frass production and beetle activity for up to 133 d, but beetle count data were truncated to 98 d in order to make uniform comparisons of beetle emergence across all bolts. All rearing occurred indoors at the University of Hawai'i at Mānoa Komohana Research and Extension Center in Hilo, Hawai'i at  $\sim 25^{\circ}\text{C}$ .

Frass was collected at least once a week from BDT and tested for the presence of viable *Ceratocystis* propagules using a modified version of the carrot-bait method developed by Moller and DeVay (1968). Frass was placed between two  $\sim 0.5$ -cm carrot discs, secured with Parafilm M (Bemis Company, Inc., Oshkosh, WI), and incubated in resealable plastic bags at room temperature. Samples were moistened with sterile water if necessary and checked for mycelium and perithecial growth characteristic of *Ceratocystis* weekly for 4 wk. To confirm morphological identifications, a subset of carrot-baits was subcultured onto 10% V8 agar media amended with 50 mg/ml streptomycin (Marin et al. 2003, Keith et al. 2015) and incubated at room temperature for 14 d. After incubation, an agar plug was collected from each subculture, placed into a 1.5-ml screw-cap tube, and stored at  $-20^{\circ}\text{C}$  until further processing. DNA was extracted from agar plugs using the NucleoSpin (Macherey-Nagel, Bethlehem, PA) Plant II kit from Machelary-Nagel and *Ceratocystis* species were identified using the cerato-platanin qPCR developed by Heller and Keith (2018).

In addition, 28 BDT were attached to active galleries in order to specifically quantify rates of frass production by beetle species. Frass was collected, dried at  $50^{\circ}\text{C}$  for 2 d, and weighed. Rate of frass production was calculated per sample by dividing the frass dry mass by the number of days elapsed since the previous collection of that BDT. All data and metadata associated with this manuscript are available at <https://doi.org/10.5066/P9RJKOO6> (Roy et al. 2020).

## Statistical Analysis

All statistical analyses were performed in R version 4.0 (R Core Team 2020). We tested whether the different beetle species showed a homogenous distribution across elevations using a Fisher's exact test of independence (FET). We analyzed whether different beetle species utilize different tree heights by fitting a linear mixed-effects model (LMM). For the model, we used beetle species as the predictor and the log(bolt height) as the response with tree as the random effect using the lmer function provided by the lme4 package for R (Bates et al. 2015). Post hoc tests for differences between model coefficients were used to compare linear combinations of tree height niche dimensions for each ambrosia beetle species using the mlcomp package (Hothorn et al. 2008). Logically constrained multiplicity adjustments were used to control multiple comparison *P*-values (Westfall et al. 1999).

We fit a binomial generalized linear mixed-effects model (GLME) using the glmer function in the lme4 package (Bates et al. 2015) to test if viable ROD-causing *Ceratocystis* was correlated to tree height and/or elevation, using log(bolt height) and log(elevation) as the predictors and viable *Ceratocystis* propagules embedded in frass as the response, controlling for tree as a random effect. A Kruskal–Wallis test by ranks was used to compare the distribution of frass produced by species, followed by the post hoc Dunn's test for multiple comparisons using the FSA package for R (Ogle 2016). Frass viability and production estimates are reported below as means  $\pm$  SE. Statistical significance was defined as  $P \leq 0.05$ .

## Results

### Beetle Community

All trees used in this study were infected with *C. lukuobia*; three trees were co-infected with *C. huliobia*, one each from Hawaiian Acres, Waiākea Forest Reserve, and Waipunalei. We reared and identified *Cryphalus longipilus* Schedl, *Xyleborinus* (*Xi.*) *andrewesi* (Blandford), *Xyleborinus* (*Xi.*) *saxesenii* (Ratzburg), *Xyleborus affinis* Eichhoff, *Xyleborus ferrugineus*, *Xyleborus perforans*, *Xyleborus simillimus* Perkins, and *Xylosandrus* (*Xa.*) *morigerus* (Blandford) from ROD-infected 'ōhi'a across Hawai'i Island (Table 1). Other beetles occasionally collected from bolts included species of Trogossitidae and Laemophloeidae.

*Xyleborinus saxesenii* was the most abundant species, comprising 80% of the total collections, followed by *X. perforans* (9%) and *X. ferrugineus* (6%) (Table 1). *Xyleborinus saxesenii* and *X. ferrugineus* were the most widely collected species across Hawai'i Island. Both species were reared from 14 of the 15 trees in this study, although *Xi. saxesenii* was reared from more than twice as many bolts

as *X. ferrugineus* (Table 1). Both *X. perforans* and *X. affinis* were reared from five sites and from nine and seven trees, respectively. We reared *X. simillimus* from all high elevation (1,000 m and above) trees. The relative abundances of *Cr. longipilus*, *Xi. andrewesi*, and *Xa. morigerus* were less than 1% and were consequently removed from tree niche dimension and elevation analyses. *Xyleborinus andrewesi* was found only at Hōlualoa, *Cr. longipilus* at North Kohala, and *Xa. morigerus* at both Kalōpā State Park and Kohala.

Beetle species distributions differed significantly by elevation ( $P < 0.01$ , FET). Neither *X. ferrugineus* nor *Xi. saxesenii* collections were restricted by elevation and were collected from 209 to 1,131 m (Fig. 3). *Xyleborus affinis* was similarly collected from a wide elevational range from 321 to 1,131 m. In contrast, *X. perforans* was collected at 730 m and below, and the endemic beetle, *X. simillimus*, was limited to the highest elevation sites 1,131 m and above.

Both *X. ferrugineus* and *X. simillimus* were reared only from the lower portions of the trees, 3.8 and 3.5 m and below, respectively (Fig. 4). Both *Xi. saxesenii* and *X. perforans* occupied similar generalized tree height niche dimensions, colonizing the entire tree bole (0.3–13.0 and 0.3–12.8 m, respectively; Fig. 4). According to our LMM, the tree height niche dimension occupied by *X. ferrugineus* was significantly different from *Xi. saxesenii* ( $\beta = -0.92 \pm 0.25$ ,  $z = -3.64$ ,  $P < 0.01$ ), and trending towards significantly different from *X. perforans* ( $\beta = -0.83 \pm 0.32$ ,  $z = -2.65$ ,  $P = 0.06$ ) (Supp Table S1 [online only]). *Xyleborus affinis* was distributed along the trees more widely than *X. ferrugineus* and *X. simillimus* but it was not collected above 6.9 m.

### Frass Viability and Production

Of the 178 individual beetle galleries tested, 37% (65/178) produced frass containing viable propagules on the first test and 52% (93/178) produced viable propagules at least once over two or more tests. We were able to identify the beetle species from 124 galleries with BDT and found that *X. ferrugineus*, *X. simillimus*, *X. perforans*, *X. affinis*, and *Xi. saxesenii* all produced frass with viable *Ceratocystis* propagules (Table 2). The percentage of total frass samples containing viable *Ceratocystis* propagules and the maximum number of days frass was collected varied by species (Table 2).

We found no support for effects of tree height and elevation on the viability of ROD-causing propagules using the GLME (height  $\beta = -0.68 \pm 0.42$ ,  $z = -1.63$ ,  $P = 0.10$ ; elevation  $\beta = -0.84 \pm 0.78$ ,  $z = -1.09$ ,  $P = 0.28$ ). Therefore, we accept the null hypothesis that there is no correlation with bolt height and/or elevation and viable *Ceratocystis* propagules in frass. Viable propagules were found in frass from the lowest tested point of 0.3 m to the highest tested point at 13 m of tree boles. Prevalence of viable *Ceratocystis*

**Table 1.** Relative abundance (% of total) and distribution of ambrosia beetles reared from ROD-infected 'ōhi'a bolts on Hawai'i Island

Ambrosia beetle species	Native home range	Total no. beetles collected	Percent relative abundance	Number of sites collected (N = 8)	Number of bolts collected (N = 87)
<i>Cryphalus longipilus</i>	Asia	3	<1	1	1
<i>Xyleborinus andrewesi</i>	Old World Tropics	62	<1	1	5 <sup>a</sup>
<i>Xyleborinus saxesenii</i>	Asia	6261	80	7	58
<i>Xyleborus affinis</i>	Neotropical	148	2	5	11
<i>Xyleborus ferrugineus</i>	Neotropical	476	6	6	24
<i>Xyleborus perforans</i>	Tropical Asia	721	9	5	17
<i>Xyleborus simillimus</i>	HI Isl. endemic	106	1	2	9
<i>Xylosandrus morigerus</i>	Tropical Asia	19	<1	2	4

<sup>a</sup>*Xyleborinus andrewesi* was reared from an emergence chamber containing five bolts.

propagules varied from tree to tree; we collected frass containing viable *Ceratocystis* propagules in all but one of the 15 trees tested, and 5 trees produced viable propagules at all heights tested along the entire bole (Table 3). Sites also varied in frequency of ROD-positive frass, and all of the bolts that produced frass from the Waipunalei site contained viable ROD-causing propagules (Table 3). All media subcultures were positive for *Ceratocystis* DNA.

A total of 22 beetle galleries were used for estimating frass production: 6 *Xi. saxesenii* with 25 collections, 12 *X. ferrugineus* with 56 collections, and 4 *X. simillimus* with 17 collections. *Xyleborus*

*ferrugineus* produced the most frass ( $7.82 \pm 1.08$  mg/d, ranging from 0.06 to 30.97 mg/d), followed by *Xi. saxesenii* ( $3.82 \pm 0.84$  mg/d ranging from 0.06 to 15.70 mg/day), and *X. simillimus* ( $1.71 \pm 0.24$  mg/d ranging from 0.16 to 3.67 mg/d) (Fig. 5). Frass production was statistically different between beetle species ( $H = 10.62$ ,  $df = 2$ ,  $P < 0.01$ ). *Xyleborus ferrugineus* produced significantly more frass than *X. simillimus* ( $z = 3.00$ ,  $P < 0.01$ ), and trended towards significantly more frass than *Xi. saxesenii* ( $z = 2.03$ ,  $P = 0.06$ ). Frass production of *X. simillimus* and *Xi. saxesenii* were not significantly different ( $z = -1.09$ ,  $P = 0.27$ ).

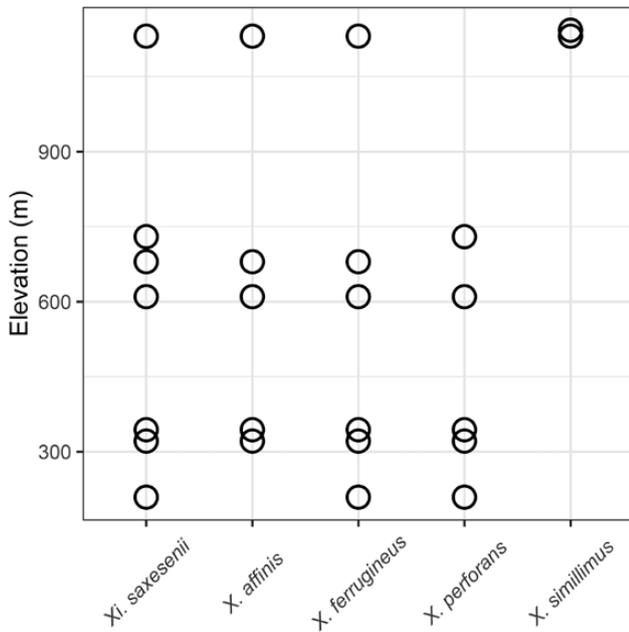


Fig. 3. Elevation of study sites where ambrosia beetle species were collected. Each circle represents one of the eight study sites.

## Discussion

### Ambrosia Beetle Species Associated With ROD

We reared eight species of ambrosia beetles from ROD-infected ‘ōhi‘a trees across Hawai‘i Island. All four of the most abundant non-native beetles are globally distributed invasives (Biedermann et al. 2012, Gohli et al. 2016). The most abundant beetle reproducing in ROD-infected ‘ōhi‘a was *Xi. saxesenii* (Table 1), which is also the most abundant ambrosia beetle in the United States (Rabaglia et al. 2019). *Xyleborus perforans*, also known as the island pinhole borer, was the second most abundant beetle in this study, and one of the first scolytids to be reported in Hawai‘i (Samuelson 1981). *Xyleborus ferrugineus* has been considered to be the most destructive ambrosia beetle in tropical forests (Wood 1982), and in our study, it was the third most abundant beetle in addition to being as widespread as *Xi. saxesenii*. The effect, if any, of *C. lukuohia* and *C. huliobia* on the reproduction of these species is yet to be determined, although it is important to note that pathogens can have a neutral or even positive effect on reproduction of ambrosia beetles, such as in the case of laurel wilt and *Xyleborus bispinatus* (Menocal et al. 2018a). The reproductive success of these beetles in ROD-infected ‘ōhi‘a in comparison to non-diseased ‘ōhi‘a and other host plants may further our understanding of the association of ambrosia beetles in the ROD pathosystem.

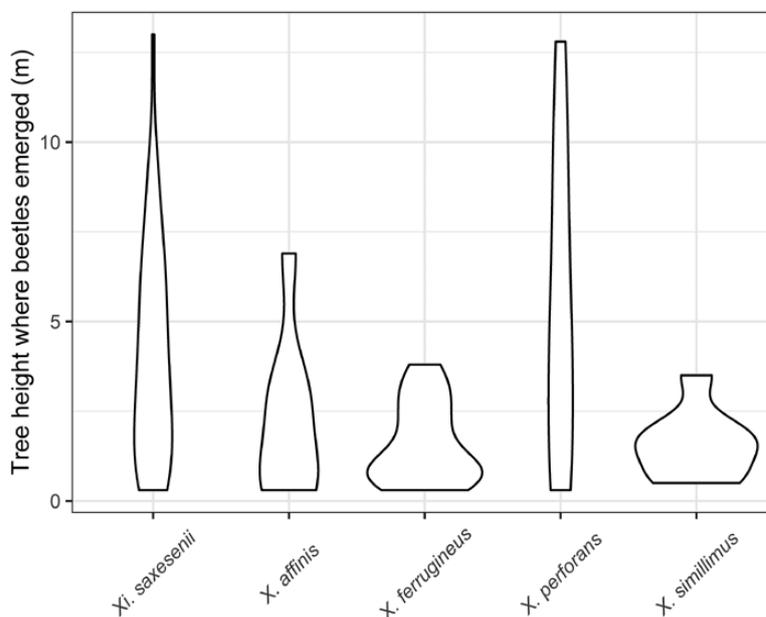
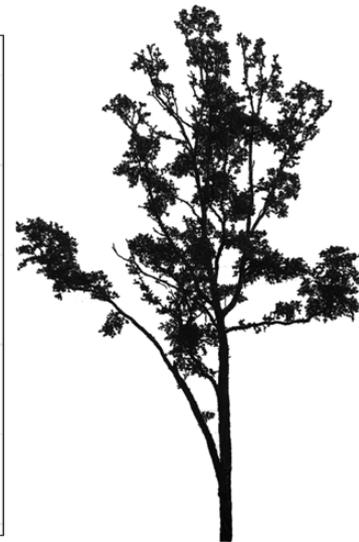


Fig. 4. Violin plots of beetle tree height niche dimension based on bolt height. Horizontal width is indicative of the number of bolts containing the beetle species at the corresponding height. ‘ōhi‘a graphic designed by Dan Mikros.



All of the most abundant and invasive ambrosia beetle species found in this study are generalists and can breed in a wide variety of hosts (Samuelson 1981, Atkinson and Peck 1994) in addition to being associated with other plant diseases. *Xyleborus ferrugineus*, *Xi. saxesenii*, and *X. affinis* are all capable of directly transmitting *Raffaelea lauricola*, the pathogen responsible for laurel wilt (Carrillo et al. 2014). Iton (1966) discovered *X. ferrugineus* was attracted to *C. cacaofunesta*-infected cacao and Herrera and Grillo Ravelo (1989) found the beetle species to be associated with *C. fimbriata* of African tulip. *Xyleborus affinis* has also been associated with *C. fimbriata* of African tulip as well as mango (Herrera and Grillo Ravelo 1989, Souza et al. 2013). *Xyleborus affinis* and *X. perforans* are both associated with Quick Tree Decline of macadamia trees in Hawai'i (Oil et al. 1991), which was linked to *Phytophthora capsici* (Ko and Kunitomo 1994). *Xyleborus perforans* is also associated with pomgranate wilt in India, another disease caused by *C. fimbriata* (Misra et al. 2020). The generalist host-plant associations of these beetles and pathogens in tropical areas are indicative of the destruction they are capable of in various plant species of differing environments.

Of the eight species of ambrosia beetles found in this study, only *X. ferrugineus* and *X. perforans* were previously associated with ROD (Roy et al. 2019) and *Xi. saxesenii* and *X. simillimus* had previously been associated with 'ōhi'a (Samuelson 1981, Stein 1983). Interestingly, *Xylosandrus crassiusculus* was documented to be associated with 'ōhi'a in the past (Samuelson 1981, Stein 1983), although we did not rear any in this study. However, previous collections were from tanglefoot traps on 'ōhi'a, and not direct rearing. Cognato and Rubinoff (2008) confirmed established *Xa. morigerus* populations on Hawai'i Island; however, this is the first record of these beetles at

multiple collection localities on Hawai'i Island in addition to being reared from 'ōhi'a. *Xyleborinus andrewesi* was recently discovered on Hawai'i Island (Cognato and Rubinoff 2008), and our results confirm an established population in the Kona district.

Because this study's focus was on ambrosia beetle's tree height niche dimension along the boles of ROD-infected 'ōhi'a, we did not characterize the beetles inhabiting the crowns and outer bark of the trees. Other beetles such as *Nesolathrus* spp. (Coleoptera: Mycetophagidae), which has been reared from ROD-infected 'ōhi'a twigs (Samuelson and Matsunaga 2019), are not reported here. However, we might expect the most common species to be more abundant in the main stems than crowns such as Reed et al. (2015) found for *X. affinis* and *Xi. saxesenii* in artificially stressed Black Walnut (*Juglans nigra*) trees. At least six native long-horned beetle species (Coleoptera: Cerambycidae) have previously been associated with 'ōhi'a (Stein 1983). Curiously, we observed some evidence of vacated cerambycid galleries in bolts from the Waipunalei and Kohala sites, but no beetles emerged. Whether cerambycids play a role in the spread of ROD is not known, and may be of importance on the leeward side of the island which was not thoroughly characterized in this study, in areas such as the Kahuku Unit of Hawai'i Volcanoes National Park and Kaloko of the Kona District (K. Roy, personal observation). Although obscure species may have not been included in this study, our data reveal the most common ROD-associated beetles, especially those most likely to spread ROD through contaminated inoculum in the form of frass.

We found distinct patterns in the altitudinal distribution of ambrosia beetle species on Hawai'i Island. The most notable elevation differences in altitudinal range were observed for *X. simillimus*, limited to high elevations 1,131 m and above, and *X. perforans*, limited to elevations 730 m and below (Fig. 3). It is possible that these species may overlap in distribution at elevations between 730 and 1,131 m, but the altitudinal gap in our collections make it impossible to determine this. Previously, Samuelson (1981) documented widespread populations of *X. simillimus* on Hawai'i Island, from elevations as low as 335 m in the Puna District to 1,280 m in Hilo Forest Reserve. Hawaiian *Xyleborines* may be vulnerable to displacement by invasive species because they tend to exhibit strong host specificity, a trait generally uncharacteristic of the group (Cognato et al. 2018). Of the 21 described native Hawaiian species of *Xyleborus* (Samuelson 1981), *X. simillimus* is particularly vulnerable being found only on Hawai'i Island and the only native beetle to host 'ōhi'a. Our collections indicate that this species may have undergone significant population declines and range retractions and now appear to remain either at very low levels or not at all in lower elevations, although further investigation is necessary to confirm the species' status. The use of high-elevation habitat as refuge from invasive species is not new to the ecology of the Hawaiian Islands, and similar patterns have been well documented in native forest birds (Atkinson et al. 2014, Paxton et al. 2016). Our data raise the potential of niche competition between *X. ferrugineus* and *X. simillimus*, which are both restricted to lower portions of trees.

**Table 2.** Ambrosia beetle species that produced frass containing viable *Ceratocystis* propagules

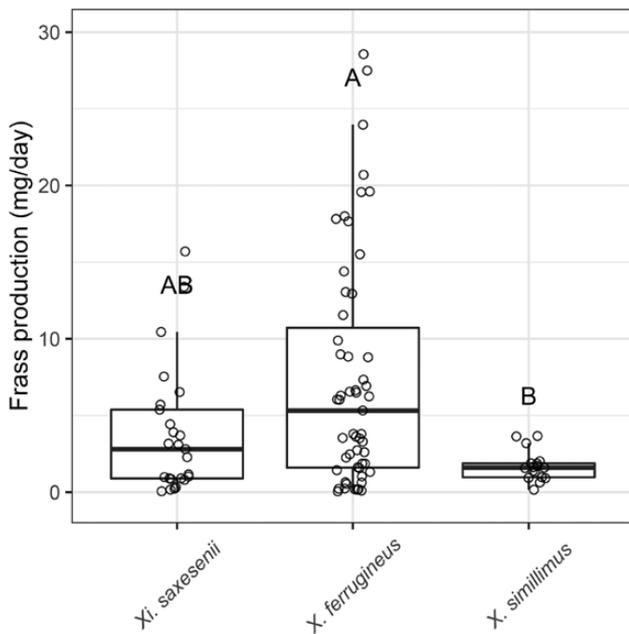
Ambrosia beetle species	Proportion of galleries that produced viable <i>Ceratocystis</i> propagules	Maximum no. of days frass collected	Total viable tests
<i>Xyleborinus saxesenii</i>	(30/47) 64%	91	(54/146) 37%
<i>Xyleborus affinis</i>	(2/3) 67%	59	(7/17) 41%
<i>Xyleborus ferrugineus</i>	(27/46) 59%	126	(102/216) 47%
<i>Xyleborus perforans</i>	(1/9) 11%	24	(1/18) 6%
<i>Xyleborus simillimus</i>	(8/19) 42%	133	(9/62) 15%

The maximum number of days frass was collected from a species' gallery does not reflect the life of the gallery but rather the maximum time laboratory space allowed frass collection.

**Table 3.** Number and percentage of bolts that produced viable *Ceratocystis* propagules from individual 'ōhi'a trees

Tree no.	Orchidlands estates	Hawaiian acres	Waiākea Forest Reserve	Waipunalei	Kohala
1	(3/5) 60%	(3/5) 60%	(2/5) 40%	(3/3) 100%	(0/3) 0%
2	(5/5) 100%	(4/5) 80%	(2/4) 50%	(4/4) 100%	(3/4) 75%
3	(2/4) 50%	(5/5) 100%	(1/2) 50%	(4/4) 100%	(2/3) 67%

Five bolts were collected from each tree, and in some cases bolts did not produce any frass.



**Fig. 5.** Boxplots describing the rate of frass produced (mg/d) by *Xi. saxesenii*, *X. ferrugineus*, and *X. simillimus* within ‘ōhi‘a bolts maintained in the lab. Different letters above boxplots indicate significant differences in frass production among species determined by the Dunn’s test of multiple comparisons following a significant Kruskal–Wallis test. Open circles represent each frass collection, whiskers represent the minimum and maximum observations, and the box represents the first quartile to third quartile, with the median depicted by a bolded black line inside the box. *Xyleborus ferrugineus* produced significantly more frass than *X. simillimus* ( $P < 0.01$ ), and approached significance with *Xi. saxesenii* ( $P = 0.06$ ). Frass production of *X. simillimus* and *Xi. saxesenii* were not significantly different ( $P = 0.27$ ).

*Xyleborus ferrugineus* was reared from two trees (one bolt each) at high elevation where *X. simillimus* was also reared. Based on similarity in the studied tree height niche dimensions and evidence of changing distribution, future work could evaluate whether *X. simillimus* populations may be in decline due to invasion by the invasive *X. ferrugineus*, a pattern that has been previously hypothesized for the Hawaiian Xyleborines (Cognato and Rubinoff 2008, Kuo 2010, Cognato et al. 2018).

The observed differences in tree height niche dimensions or position along tree boles for *Xi. saxesenii* and *X. ferrugineus* may indicate niche partitioning between the species in order to reduce interspecific competition. Behavioral differences such as flight capacity, aggressiveness, and/or attractiveness to kairomones may all play a role in niche partitioning. Menocal et al. (2018b) trapped *Xi. saxesenii* and *X. affinis* flying at three height stratifications (0–2, 2–4, and 4–6 m), similar to the brood distribution we saw in ROD-infected ‘ōhi‘a. The lower portion of trees may be more desirable due to moisture limitations required for the proliferation of ambrosia fungi, furthering reproductive success and delayed dispersal (Rudinsky 1962), and may explain the restriction to basal areas of trees seen in *X. ferrugineus* and *X. simillimus*. Alternatively, kairomones may be emitted at higher concentrations in the trunk of ROD-infected ‘ōhi‘a, similar to the sesquiterpenes emitted from avocado (Niogret et al. 2013), particularly because *X. ferrugineus* has been documented to be attracted to the congeneric *C. cacaofunesta* (Iton 1966).

## The Importance of Frass in the ROD Pathosystem

The variability in viable *Ceratocystis* propagules produced from individual galleries does not appear to be dependent on beetle species, and any species excavating a gallery in infected wood tissue may be capable of releasing viable *Ceratocystis* propagules into the environment regardless of life history. For example, although *Xi. saxesenii* larvae are xylomycetophagous (feeding on both fungi growing on wood and the wood itself) (Deyrup and Atkinson 1987) and the passing of the wood through the gut could potentially kill any embedded fungal propagules, all life stages of *Xi. saxesenii* co-occur in the gallery system and any material extruded into the environment by the larvae would also include frass of adults (Hosking 1973, Biedermann et al. 2012). Instead, viability may be dependent on fungal growth at the excavation location, particularly because galleries of Xyleborini beetles often extend through both the sapwood and heartwood of trees (Fig. 1D; Hosking 1973, Samuelson and Gressitt 1976, Roeper et al. 1980, Peer and Taborsky 2007, Misra et al. 2020), although further investigation is necessary. Hughes et al. (2020) recently found that *C. lukuohia* can penetrate the heartwood of ‘ōhi‘a, further increasing the potential for beetles to contact the fungus during gallery excavation.

Xyleborini often host a single natal gallery for years due to the high fitness cost of dispersal (Schneider 1987; Peer and Taborsky 2007; Biedermann et al. 2011, 2012), and therefore viable inoculum in the form of frass could be released into the environment for an extended period of time. Because the exact time of inoculation was unknown for the ‘ōhi‘a trees in this study, we do not know the age of the galleries or how long the beetles had been releasing viable *Ceratocystis* propagules into the environment. However, we do know that beetles can emit viable propagules for at least 133 d (Table 2).

We found no evidence that tree height and elevation have significant effects on the presence of viable *Ceratocystis* propagules in frass. Instead, viability may be dependent on other factors such as physical characteristics of the tree itself, time since tree infection, or abiotic factors including temperature, humidity, and rainfall. Additional work that combines artificial inoculations with long-term beetle monitoring could help to identify the relative importance of these factors.

We present measurements of frass production in *Xi. saxesenii*, *X. ferrugineus*, and *X. simillimus* while brooding in *Ceratocystis*-infected ‘ōhi‘a in the lab for the first time. We recognize that the number of progeny in each gallery may be different due to delayed dispersal (Biedermann et al. 2011, 2012), and as such our data realistically represent the patterns of frass production in the wild but do not capture per-individual beetle rates. We present the first attempt to quantify the effect these beetles can make on the environment in terms of inoculum. We found that *X. ferrugineus* produces over 4x more frass than *X. simillimus* and is more likely to play a significant role in inoculum spread than the native (Fig. 5). Frass production can be an indicator of colony health (Menocal et al. 2018a), and the copious rate of frass produced by *X. ferrugineus* may be indicative of their reproductive success, raising the potential of further rapid population growth and additional detrimental effects on Hawaiian species and ecosystems. Because of the lower tree heights occupied by *X. ferrugineus* and *X. simillimus*, their frass may be more likely to be transported via soil and less likely to be dispersed by wind over long distances than that of *Xi. saxesenii*, which was found throughout the tree bole. We do not have frass production data for either *X. affinis* or *X. perforans*, and they occupy relatively high areas on ROD-infected ‘ōhi‘a trees. However, we can speculate that their frass production rate may be similar to that of *X. ferrugineus* due to their

invasive status and similar body size. Although Koch's postulates have not yet been filled for frass contaminated with *Ceratocystis* in this system, frass produced by *X. ferrugineus* containing *C. cacaofunesta* propagules has been shown to kill cacao seedlings (Iton 1961). We tracked nearly 200 galleries in this study, which was only a fraction of the active galleries present, and found that each active gallery could produce considerable amounts of viable *Ceratocystis* propagules. Because of the substantial amount of contaminated frass that could be expelled into the environment every day, the development of management strategies to reduce the amount of frass in the environment may be prudent to control the spread of ROD.

### Conclusions and Management Implications

We found that ambrosia beetles colonize ROD-infected trees and produce copious amounts of frass containing viable *Ceratocystis* propagules along the entire length of 'ōhi'a tree boles. These data reveal that the application of insecticides to standing trees from the ground would be ineffective, as tree heights above 2 m could not be reached with backpack sprayers. Furthermore, ROD occurs in forested areas where endemism of non-target invertebrates is high (Gruner 2004), and many of the ROD-infested areas on Hawai'i Island contribute to watershed systems (Fortini et al. 2019). Alternatively, felling and removal of infectious material have been proven to control the spread of many bark and ambrosia beetle-related tree pathogens (Billings 2011, Tsopelas et al. 2017). However, complete removal of slash in forested settings are logistically impossible, and the exploration of tarping and solarization could reduce brood survival and ultimately alter moisture conditions of the logs, further reducing fungal viability (Ollieu 1969, Krawczyk et al. 1982, Jones and Paine 2015). Contaminated ambrosia beetle frass is likely the most important pathway for potential *Ceratocystis* inoculum to be released in the environment. However, mechanical wounding by humans, ungulates, insects, and/or extreme weather events is necessary for contaminated frass to infect a healthy 'ōhi'a tree (Atkinson et al. 2019), and mechanisms to prevent wounding such as fencing and avoiding pruning could slow the spread of the disease (Keith et al. 2020). Although ambrosia beetle frass has been linked to many *Ceratocystis* diseases, (Iton 1961, Ocasio-Morales et al. 2007, Harrington 2009, Souza et al. 2013, Roy et al. 2019), the literature lacks in-depth investigations and solutions to prevent the spread of these detrimental pathogens through the frass pathway.

### Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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