

# Tree resin flow dynamics during an experimentally induced attack by *Ips avulsus*, *I. calligraphus*, and *I. grandicollis*

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**Abstract:** The success of tree colonization by bark beetles depends on their ability to overcome host tree defenses, including resin exudation and toxic chemicals, which deter bark beetle colonization. Resin defenses during insect outbreaks are challenging to study in situ, as outbreaks are stochastic events that progress quickly and thus preclude the establishment of baseline observations of non-infested controls. We use synthetic aggregation pheromones to demonstrate that confined *Ips* bark beetle herbivory can be successfully initiated to provide opportunities for studying interactions between bark beetles and their hosts, including the dynamics of constitutive and induced resin exudation. In *Pinus taeda* L. plantations between 12 and 19 years old in North and South Carolina, U.S., trees were affixed with pheromone lures, monitored for evidence of bark beetle attacks, and resin samples were collected throughout the growing season. Baiting increased beetle herbivory to an extent sufficient to produce an induced resin response. Attacked trees exuded about three times more resin at some time than control trees. This supports previous work that demonstrated that information on constitutive resin dynamics alone provides an incomplete view of a host tree's resistance to bark beetle attack.

**Key words:** bark beetle outbreak, pheromones, *Pinus taeda*, plant defense, resin flow.

**Résumé :** Le succès de la colonisation des scolytes dépend de leur capacité à surmonter les défenses de l'hôte, incluant l'exsudation de résine et de composés chimiques toxiques qui empêchent la colonisation des scolytes. La production de résine comme mécanisme de défense lors des épidémies d'insectes est difficile à étudier in situ étant donné que les épidémies sont des événements aléatoires qui progressent rapidement et excluent par conséquent l'établissement d'observations de référence chez des témoins non infectés. Nous utilisons les phéromones d'agrégation synthétiques pour démontrer que l'herbivorie confinée des scolytes du genre *Ips* peut être initiée avec succès pour fournir l'occasion d'étudier les interactions entre les scolytes et leurs hôtes, incluant la dynamique de l'exsudation constitutive et induite de résine. Dans des plantations de *Pinus taeda* L. âgées de 12 à 19 ans, en Caroline du Nord et du Sud aux États-Unis, des arbres ont été munis d'appâts à base de phéromones et suivis pour détecter les signes d'attaques des scolytes; des échantillons de résine ont été prélevés tout au long de la saison de croissance. L'utilisation d'appâts a augmenté l'herbivorie des scolytes suffisamment pour induire la production de résine. À certains moments, les arbres attaqués exudaient environ trois fois plus de résine que les arbres témoins. Ces résultats concordent avec ceux de travaux antérieurs qui démontrent que l'information sur la dynamique de la résine constitutive seule fournit une vision incomplète de la résistance des arbres hôtes aux attaques des scolytes. [Traduit par la Rédaction]

**Mots-clés :** épidémie de scolytes, phéromones, *Pinus taeda*, mécanisme de défense des plantes, écoulement de résine.

## 1. Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are some of the most devastating insect pests in the coniferous forests of the southeastern United States (US). Their damage results in massive economic losses and changes in ecosystem structure and function and creates challenges for natural resource policy (Werner et al. 2006; Raffa et al. 2008; Price et al. 2010; Coulson and Meeker 2011; Pye et al. 2011; Meddens et al. 2012). Global climate change is predicted to result in conditions that are favorable to bark beetle fitness (Gan 2004) and range expansion into new areas and (or) new hosts (Cudmore et al. 2010; Lesk et al. 2017).

Bark beetle species vary in terms of their reproduction dynamics and how aggressively they attack trees. Attacks by Southern Pine Beetle (*Dendroctonus Frontalis* Zimmerman, heretofore SPB), for example, can quickly evolve into widespread outbreaks that kill healthy trees. Species of *Ips* bark beetles (*Ips avulsus* (Eichhoff), *Ips calligraphus* (Germar), and *Ips grandicollis* (Eichhoff)), on the other hand, generally attack stressed or weakened trees but pose a threat to healthy trees when vigor is reduced, occasionally causing tree mortality (Drooz 1985; Riley and Goyer 1988; Fettig et al. 2007; Schowalter 2012; Clarke et al. 2000). Despite these differences in reproduction dynamics and host preference, however,

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bark beetles tend to invade and colonize trees by similar mechanisms. Adult bark beetles breed, create nuptial chambers, and excavate egg galleries in the host tree's phloem and outer xylem (Raffa et al. 2015), and larvae consume phloem tissue, a process that halts the transport of carbohydrates and water. A tree can tolerate some loss of this conductive tissue, but a loss of too much conductive tissue, combined with deleterious effects of symbiotic blue stain fungi on tree xylem, can result in tree mortality. Because *Ips* bark beetles are less aggressive and more likely to attack weakened trees (Hain et al. 2011; Cognato 2015), they could be used to investigate tree defense responses under various conditions of environmental stress and study the links between tree physiology and defense against attacking bark beetles.

The exudation of resin (also called oleoresin) by pine trees represents both a physical and chemical defense against invading bark beetles. This resin can physically eject or entomb the beetles, and the containment of beetles and (or) associated fungi allows the tree an opportunity to withstand low-density beetle attacks and prevent or delay the onset of outbreaks (Knebel et al. 2008; Klepzig and Hofstetter 2011). Resin also contains monoterpenes and phenolic compounds that may be toxic to attacking beetles and their mutualistic fungi (Franceschi et al. 2005; Bohlmann 2012) and that may affect host selection (Fischer et al. 2010). Viscosity of the resin is determined by the ratio of all classes of monoterpenes, including sesquiterpenes and diterpene acids; the latter have insecticidal properties contributing to the resistance to colonization (Phillips and Croteau 1999). Chemical components and exudation rates are highly variable between individual trees, and some chemical defenses occur ancillary to resin exudation. Resin flow may be "constitutive", representing resin synthesized prior to attack, or "induced", representing newly synthesized resin after phloem wounding (Ruel et al. 1998; Lombardero et al. 2000). During active bark beetle attacks, trees can reallocate resources to actively mobilize newly synthesized (i.e., induced) resin for defense, resulting in higher exudation rates until the insect threat has passed, available resources in the tree are depleted, or the tree is otherwise compromised (Lombardero et al. 2000; Franceschi et al. 2005; Luchi et al. 2005; Knebel et al. 2008). The induced (secondary) resin contains toxic levels of monoterpenes in addition to acting as a physical barrier (Martin et al. 2002; Boone et al. 2011). Once a tree's resin defenses are overcome, the level of bark beetle colonization can lead to tree mortality (Boone et al. 2011).

Lower constitutive resin flow has been correlated with lower resistance to bark beetle attack, though mechanistic drivers of variability in both constitutive and induced resin flow are not well characterized (Lombardero et al. 2000; Knebel et al. 2008; Novick et al. 2012). Resin production and flow rate vary inter- and intraspecifically, depending on site-specific factors, including management and water availability (Hodges and Lorio 1975; Hodges et al. 1977; Franceschi et al. 2005). One popular conceptual framework for predicting constitutive resin flow responses is the growth-differentiation balance hypothesis (GDBH; Herms and Mattson 1992), which predicts a trade-off between growth and defense, particularly when trees experience mild resource deficits. Under limited resource availability such as low soil nutrients and moderate water stress, photosynthate allocation may be shifted from growth to secondary metabolites (Lorio and Sommers 1986), increasing constitutive reserves of resin (Warren et al. 1999). In contrast, when nutrient or moisture limitations are alleviated, carbohydrates are preferentially allocated to growth and constitutive resin reserves are reduced (Warren et al. 1999). Moderate water stress, however, affects the chemical composition of resin, with decreased acids and increased monoterpene hydrocarbons rendering them more susceptible to bark beetle attack (Hodges and Lorio 1975). Finally, severely drought stressed trees often demonstrate a reduction in resin production (Lombardero et al. 2000;

Gaylord et al. 2013; Netherer et al. 2015) when carbohydrate supply is highly compromised.

It is less clear, however, how well the GDBH can predict the dynamics of induced resin flow. Expression of inducible resin defenses may be limited to rapidly growing (i.e., nutrient rich) plants, suggesting that fertilization, while possibly decreasing constitutive resin reserves, may increase the ability of host trees to induce resin flow during herbivory (Herms and Mattson 1992). Complicating matters is the fact that studies on induced resin flow are less common than on constitutive flow because bark beetle outbreaks are difficult to predict and often occur quickly, thus challenging efforts to observe pine tree defense dynamics during active herbivory. Previous efforts to promote an induced resin response have included mechanical wounding and fungal inoculation techniques. These techniques have been successfully deployed to understand resin defense and bark beetle attack (Lombardero et al. 2006), environmental stress effects on terpene and phenolic accumulation (Klepzig et al. 1995), and prior wounding effects on acquired resistance induction (Christiansen et al. 1999). They are, however, labor intensive (Ruel et al. 1998; Knebel et al. 2008), and physical wounding by itself does not always promote an induced resin flow response (Lombardero et al. 2000; Klepzig et al. 2005; Luchi et al. 2005; Mason et al. 2017). Moreover, when the treatments are confined to the accessible lower portion of the bole, they cannot promote direct wounding and subsequent fungal invasion in the upper portion of the tree, where many bark beetles prefer to attack (Anderson and Anderson 1968; Birch 1978). This may unintentionally exclude important host tree – bark beetle interactions (i.e., direct severing of resin ducts in the upper branching).

Here, we explore the utility of synthetic pheromones to lure bark beetles to attack study trees with the specific goal of measuring resin flow throughout the course of an active bark beetle attack. Much previous work has relied on the use of synthetic pheromones to attract bark beetles to study a range of processes, including crown scorch effects on colonization success (Wallin et al. 2003), effect of crown density and tree vigor on host susceptibility (Waring and Pitman 1983), and aggregation behavior in response to synthetic pheromones (Vité and Pitman 1969). To our knowledge, however, none have relied on the application of pheromones to attract bark beetles with the particular goal of eliciting an induced resin flow response. Given the ecological and economic importance of bark beetles and their pine tree hosts in the southeastern US, developing a broader set of tools for inducing the study of links between host tree physiology and constitutive and induced resin defenses would improve our ability to develop effective 21st century management approaches.

The specific objective of this work was to develop an experimental baiting approach that allows us to assess the in situ response of pine trees to bark beetle attacks and to apply the approach in two experiments to better understand the temporal and spatial dynamics of resin flow during active bark beetle attack. In the first pilot experiment, our objective was to test whether the use of aggregation pheromones would successfully attract *Ips* bark beetles into a localized forested area. This could offer an opportunity to study the tree's defense response from initial beetle colonization through the establishment phase. We focused on *Ips* bark beetles because they physically bore into the phloem and xylem of trees but usually do not cause extensive outbreaks like SPB, mitigating the risk that our methods would lead to insect damage outside of the study domain.

A second experiment, designed to more precisely characterize the resin flow response to invading bark and wood-boring insects, was replicated in two study locations. Our first objective was to demonstrate whether this technique could produce an induced resin flow response to better understand and disentangle the relationship between constitutive and induced resin flow. Our second objective was to explore possible differences in the consti-

**Table 1.** Weather, abiotic variables, stand conditions, and experimental details at the two study sites used in this study.

|   | Study sites                                     |   |
|---|---|---|
|   | SRS*  | SETRES-II†  |
| County and state  | Aiken, South Carolina                           | Scotland, North Carolina                                  |
| Coordinates   | 33°N, 81°E                                      | 34°N, 79°W  |
| Annual mean temperature (°C)                                  | 18  | 17  |
| Physiographic region  | Aiken Plateau Sandhills                         | Georgia/Carolina Sandhills                                |
| Climate   | Humid continental: warm summers, mild winters   | Humid continental: warm summers, mild winters             |
| Soil series   | Blanton   | Wakulla   |
| Annual mean precipitation (mm)                                | 1320  | 1210  |
| DBH (mean (SD), cm)   | 19.75 (3.18)                                    | 16.79 (3.87)  |
| Age (years)   | 12  | 19  |
| Fertilizer treatment (-ha <sup>-1</sup> .year <sup>-1</sup> ) | 120 kg N*                                       | 79 kg N, 8 kg P, 8 kg K, 0.6 kg Ca, 5 kg Mg, and 15 kg S‡ |
| Spacing (m)   | 2.5 × 3   | 1.5 × 2.1   |
| Tree density (no. of trees·ha <sup>-1</sup> )                 | 1333  | 3076  |
| Plot size (ha)  | 0.22  | 0.25  |
| Bait deployment date  | 7 June 2012                                     | 7 April 2013  |
| Resin flow samples  | Days 1, 7, 25, 62, and 86 after bait deployment | Days 1, 15, 48, 92, 108, and 117 after bait deployment    |
| Sticky collections  | Days 10, 17, and 22 after bait deployment       | Days 48 and 92 after bait deployment                      |
| Average no. of holes  | ~16   | ~36   |
| Duration (days)   | 86  | 117   |

Note: DBH, diameter at breast height.

\*Coleman et al. 2004.

†Albaugh et al. 1998.

‡From 1994 to 2007, annual fertilizer treatments varied with this average nutrient application (S. McKeand, personal communication, 2012).

tutive and induced resin flow as a function of management (e.g., fertilization) and climatic variability. In particular, we test if non-fertilized plots demonstrate greater constitutive resin flow than fertilized plots, as suggested in the resource availability hypothesis (Coley 1987) and the growth-differentiation balance hypothesis (Herms and Mattson 1992), and further, if fertilized plots produce greater inducible resin exudates in line with the resource availability hypothesis (Coley 1987; Endara and Coley 2011). This study explores the physical aspect of pine tree resin defense from the initial onset of attack throughout herbivory and provides insight into the relationship between constitutive and induced resin response to beetle attack.

## 2. Materials and methods

### 2.1. Study sites

We conducted studies in two managed loblolly pine plantations on the Atlantic Coastal Plain in the southeastern US: one in South Carolina and one in North Carolina. Study trees were between 12 and 19 years old, and sites were situated on sandy soil with a similar climate (Table 1). As a proxy for local soil water availability, we calculated a 7-day moving window of total precipitation using data from the North Carolina Climate Retrieval and Observations Network of the Southeast (CRONOS) database (<http://climate.ncsu.edu/cronos/>) (Figs. 5e and 5f).

The pilot study and one of the replicated resin response experiments took place at the U.S. Department of Energy's Savannah River Site (SRS), a National Environmental Research Park, in Aiken County, South Carolina, in 2011 and 2012. Trees were kept weed and pest free by mowing weeds between tree rows and applying chemical pest management techniques when appropriate, including glyphosate, carbaryl, and imidacloprid. Study trees had not received any chemical pest management treatments for the previous 5 years. From planting in spring 2000 through the 2010 growing season, separate plots received either irrigation (I; 3.0 cm water·week<sup>-1</sup>), fertilization (F; 120 kg N<sup>-1</sup>.ha<sup>-1</sup>.year<sup>-1</sup>), or irrigation and fertilization (I + F) or were untreated (control) (Coleman et al. 2004; Coyle et al. 2016). The second replicated response experiment was conducted at the Southeast Tree Research and Education Site II (SETRES-II) in Scotland County, North Carolina, where fertilization plots received 79 N, 8 P, 8 K, 0.6 Ca, 5 Mg, and 15 S kg·ha<sup>-1</sup>.year<sup>-1</sup>. Control of non-pine vegetation had

been maintained since 1992 through mechanical and chemical (glyphosate) methods (Albaugh et al. 1998).

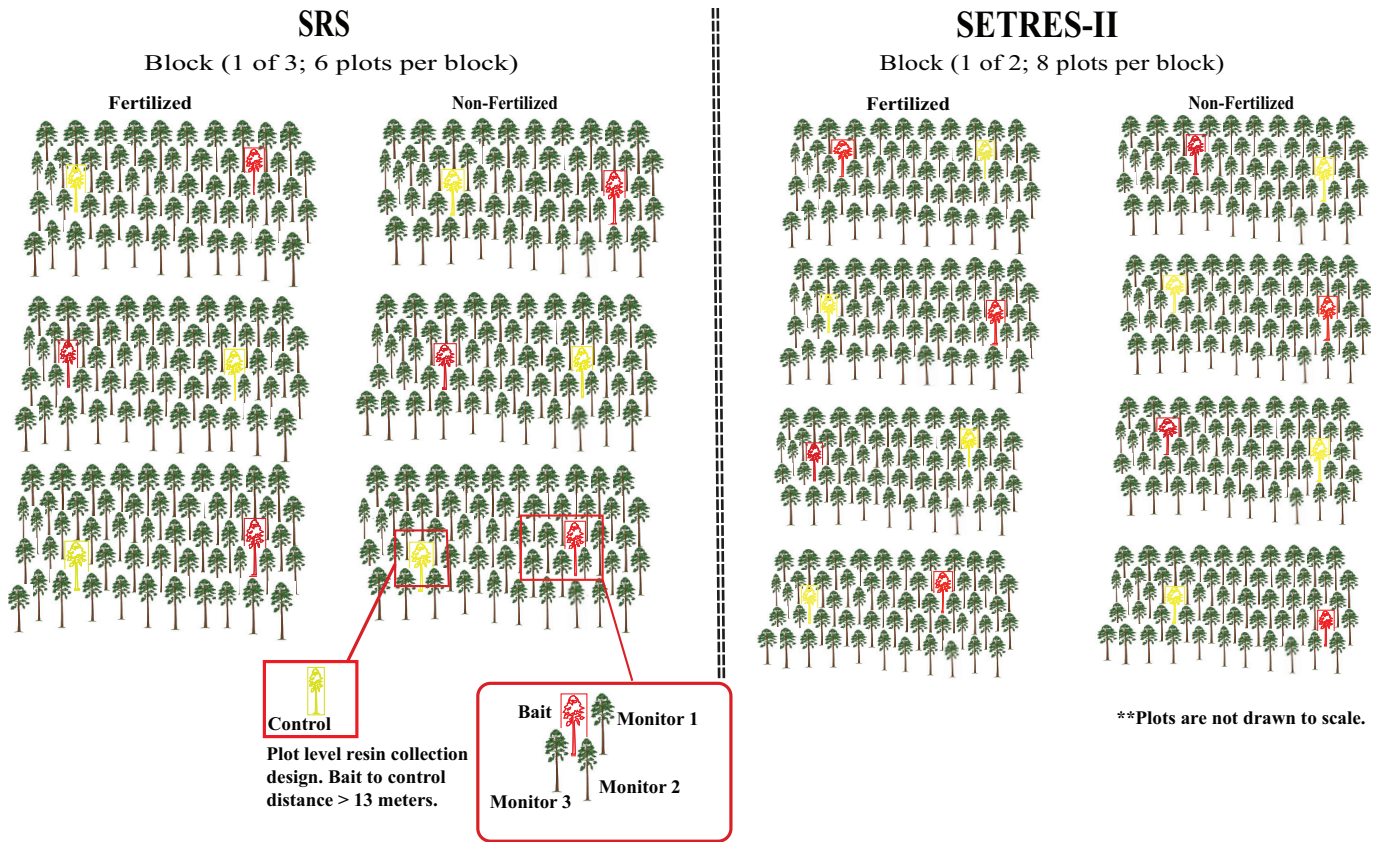
### 2.2. Pilot study at SRS

During the summer of 2011, we tested the efficacy of aggregation pheromones and tree stress volatiles to initiate a controlled infestation of *Ips* bark beetles. The experiment occurred in three plots receiving the I + F treatment (one plot in each of three blocks). In each plot, two trees, each ~25 cm diameter at breast height (DBH, 1.4 m) and separated by at least 25 m, were selected to receive bait. On 7 June 2011, we attached ipsenol (+50/-50 bubble capsule 40 mg), ipsdienol (+50/-50 bubble capsule 40 mg), and kairomones ( $\alpha$ -pinene and ethanol ultra-high release (UHR)) lures (all from Contech Enterprises, Victoria, B.C., Canada) to each tree. Baited and adjacent trees (located within 3 m of the baited trees) were monitored on 14 June, 28 June, and 23 August for holes resulting from insect entrance and (or) exit. In some cases, entrance holes had the typical pitch tube associated with a successful bark beetle attack, while in other cases, the tree was unable to produce enough resin to cause a pitch tube. Exit holes were always without a pitch tube. Because some entrance holes may have looked identical to exit holes, we were unable to separate the two hole types and thus lumped them into one metric hereafter called "bark beetle holes".

We recorded bark beetle holes (primarily from *Ips* species, though it is possible that SPBs may have been present as SPBs are known to persist in very low populations on weakened, injured, or *Ips* beetle attacked trees during their endemic phase, often indicated by the presence of sawdust on the bark or ground) and concave egg niches carved in the bark by the pine sawyers *Monochamus titillator* (Fabricius) and *Monochamus carolinensis* (Olivier) (egg niches appear identical for these two species). Yellow multi-gard sticky traps (15 × 10 cm, Scentry Biologicals Inc., Billings, Montana) that did not contain any attractants were installed, allowing for beetle identification using a microscope to ensure that no SPBs were landing. On 14 June, we estimated insect holes on the lower 3 m of the bole. We quantified these holes on three vertical locations on the bole (1.4 m, 2 m, 3 m) on 28 June (using a 13 × 18 cm square) and on two sides of the tree at DBH level and at ~3 m on 23 August (using a 22 × 16 cm square). We explored



**Fig. 1.** Experimental design for Savannah River Site (SRS), 2012 (left) and Southeastern Tree Research and Education Site II (SETRES-II), 2013 (right). [Colour version available online.]



relationships between bark beetle hole density low and high on the bole.

### 2.3. Resin response study at SRS and SETRES-II

Following a successful pilot experiment (see section 3.1), an experiment was conducted in 2012 at SRS to include a larger number of study trees to monitor insect landing and insect entrance and exit holes and to measure resin flow. The resin response experiment at SRS was designed to compare fertilized and non-fertilized treatments (Fig. 1). We established three replicated blocks, each consisting of three plots (three blocks  $\times$  two treatments  $\times$  three plots). Within each plot, 15 trees (i.e., three sets of five) were selected for a total of 90 sample trees. One tree within each set was baited with synthetic aggregation pheromones (as described in the pilot study) and three trees located adjacent ( $\geq 8$  m) to the baited tree (termed “bait-adjacent” trees) were monitored to determine the spatial extent of baiting. One additional tree more than 13 m from the bait was monitored as the control, with the expectation that the effect of baiting would not affect trees at this distance. Beetle presence in the region is widespread, and small numbers of bark beetle holes were sometimes observed in non-baited trees. We did not attempt to exclude beetles from our control trees. Instead, we leveraged information about bark beetle holes on control trees to set a baseline of damage for trees operationally classified as “attacked” as described in more detail later.

In 2013, a second resin response experiment was conducted at SETRES-II. The procedures at SETRES-II were similar to those at SRS, with fertilized and non-fertilized treatments (Fig. 1). We established two replicated blocks, each consisting of four plots (two blocks  $\times$  two treatments  $\times$  four plots). An additional fifth plot was established in one non-fertilized treatment, for a total of 17 plots (Fig. 1). Within each plot, we sampled five trees: one baited, three

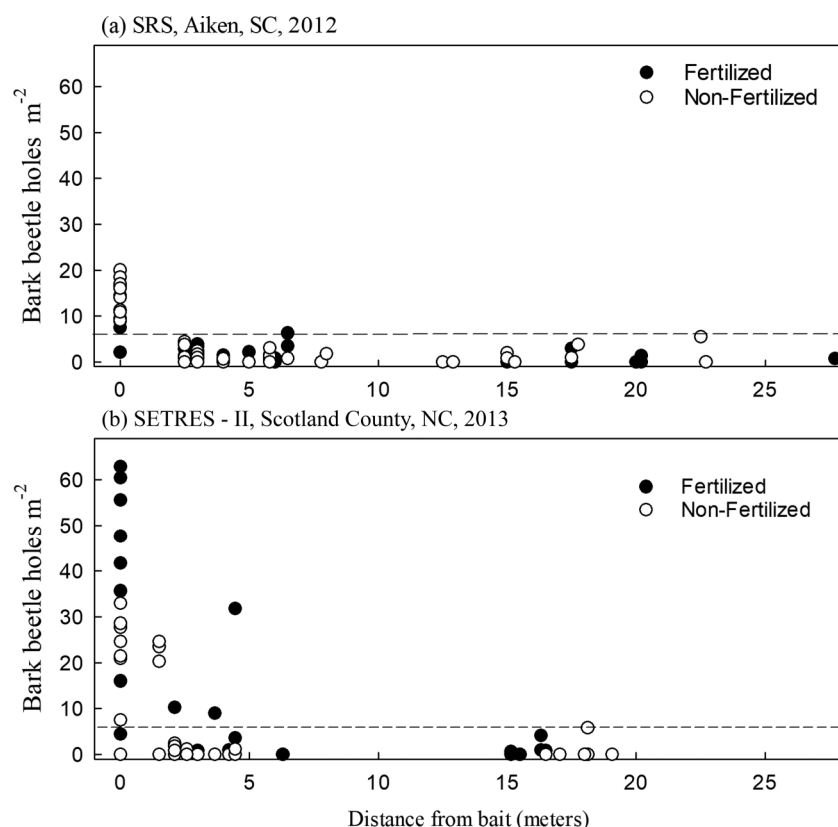
bait-adjacent, and one control more than 13 m away from baited for a total of 85 trees.

#### 2.3.1. Insect landing and bark beetle holes

During the growing season, we visually assessed bark and wood-boring beetle damage on the lower 2 m (from base to 2 m high) of each study tree using methods identical to those used in the pilot study. In the SETRES-II experiment, we also used Swedish climbing ladders to assess damage to approximately 4 m high around the entire stem. Holes by the black turpentine beetle (*Dendroctonus terebrans* (Olivier); BTB), identifiable by large resinous pitch tubes on the lower bole, were also recorded. Yellow multi-gard sticky traps (15  $\times$  10 cm, Scentry Biologicals Inc.) that did not contain any attractants were installed on all baited and control trees to quantify *Ips* bark beetle abundance. Traps were installed approximately 2.4 to 3 m from the base of the tree and mounted directly on the stem. Traps captured *Ips avulsus*, *I. calligraphus*, and *I. grandicollis*. Beetles were identified using the taxonomic key in Wood (1982) and attack density was derived from bark beetle holes. Quantification of insect damage was similar at both sites, though trap collection dates differed (Table 1).

We pooled bark beetle hole data from SRS and SETRES-II to determine the threshold of bark beetle activity beyond which trees were operationally classified as attacked. We classified all trees with six or fewer holes as non-attacked, as six was the maximum number of insect holes observed on trees located more than 13 m from the baited trees. All trees with greater than six holes were classified as attacked. Our results are not particularly sensitive to the magnitude of the threshold differentiating attacked from non-attacked trees; had the attack threshold been set at two standard deviations (SD) (nine holes) above the established level, it would have resulted in no fewer trees classified as attacked at SRS and only two fewer at SETRES-II.

**Fig. 2.** Bark beetle holes in relation to distance (m) from pheromone baits at (a) SRS and (b) SETRES-II for non-fertilized (open circles) and fertilized (solid circles) trees.



Among trees located more than 13 m from the baited tree, the mean number of observed bark beetle holes in the lower 2 m of the bole was 0.95 (SD = 1.6), ranging from 0 to 6 (Fig. 2). We selected the maximum number of bark beetle holes on these trees, which represents six times the mean number of holes, to represent the threshold level for ambient beetle activity in non-attacked trees. In contrast, mean beetle holes on baited trees were 21.9 (SD = 15.2), ranging from 0 to 64 (Fig. 2). At SRS, 18 of the 18 baited trees (100%) exceeded the threshold and were considered attacked, whereas no bait-adjacent trees were attacked. Baited trees at SETRES-II had a greater total number of trees attacked than at SRS. The majority of baited trees exceeded the attack threshold (14 of 17 trees, or 82%) plus five of 51 bait-adjacent trees were characterized as attacked (~10%). We emphasize that the majority of bait-adjacent trees were not attacked.

### 2.3.2. Volumetric observations of resin flow

Resin flow sampling began when trees were baited and continued through multiple sampling events over the rest of the growing season (Table 1). On each sampling date, resin flow rate ( $\text{g}\cdot\text{day}^{-1}$ ) was measured in the five trees that were selected to be monitored as bait, bait-adjacent, and control, with two samples per tree collected from opposite sides of the stem. Following standard practice (Lombardero et al. 2000; Knebel et al. 2008; Novick et al. 2012), resin flow was measured by removing a 0.8 cm diameter disk of bark to the phloem-xylem interface at DBH and installing resin samplers around the wound to direct the flow of resin into preweighed collection vials for a  $24 \pm 1.5$  h period. Vials were installed on all trees between 0800 and 1200 to minimize time-of-day effects on resin flow. Measurements were repeated on the same set of trees and care was taken to ensure that new wounds were laterally displaced 5–10 cm from previous wound sites. Once collected, tubes were sealed and taken to the labora-

tory to be weighed. Resin flow collection methodology was identical at each site; however, on day 15 after baiting during the SETRES-II field campaign, the site experienced an extreme rain event that contaminated the resin flow vials; those data were removed from the analysis.

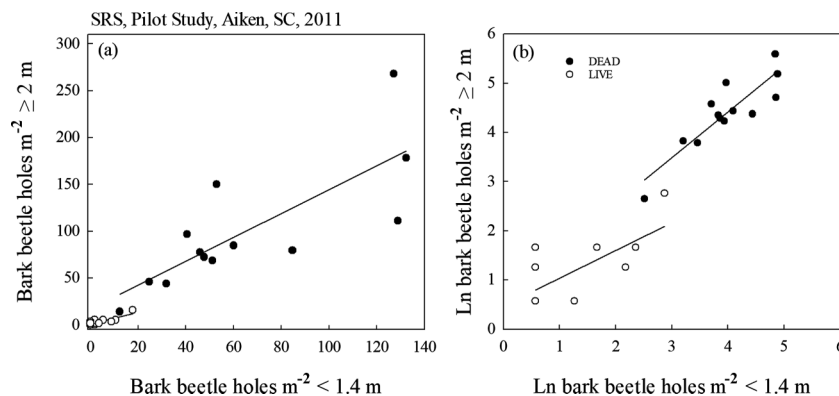
### 2.3.3. Statistical analysis

Statistical analysis was performed using SAS 9.3 (SAS Inc., Cary, North Carolina, USA) and we considered an  $\alpha$  level of 0.05 significant for all analyses. A random effects mixed model analysis of variance (ANOVA) was used to test for the effects of fertilization treatment and baiting for bark and wood-boring beetles on amount of resin. The model for fixed effects, including interaction terms, took the following form:

$$(1) \quad Q = \mu + D + T + A + DT + DA + TA + DTA + \varepsilon$$

where  $Q$  is the resin flow metric of interest,  $\mu$  is the overall mean resin flow,  $D$  is the day effect (categorical variable),  $T$  is the treatment effect (non-fertilized or fertilized),  $A$  is the beetle attack (attacked or not attacked), and  $\varepsilon$  is the error associated with the model with expectations of normal independent distribution (NID;  $\sim 0, \sigma_\varepsilon^2$ ). Random effects were assigned based on the nested design of trees (individuals) within a plot (replicate) within a fertilization treatment within a block. Statistical analysis was performed separately because the sites were of different age and size, and studies were performed in different years with varying climate conditions. Both sites were *Pinus taeda* plantation sites; however, sites used different provenances of the species, as well as having different applications of fertilizer treatments and controls of understory growth.

**Fig. 3.** Regression for insect holes observed below 1.4 m and above 2 m using data from our pilot study: (a) insect holes for live (open circles) and dead (solid circles) trees ( $R^2 = 0.88$ ,  $A_{\text{high}} = 1.45A_{\text{low}} + 0.54$ ,  $P < 0.01$ ); (b) log transformation of insect holes for live and dead trees ( $R^2 = 0.90$ ,  $A_{\text{high}} = 1.10A_{\text{low}} - 0.091$ ,  $P < 0.01$ ).



We analyzed several resin flow metrics in an effort to understand treatment effects on both constitutive and induced resin flow. We compared treatment effects on flow collected prior to baiting ( $Q_{\text{initial}}$ ) and on the final sampling day of each experiment ( $Q_{\text{final}}$ ). Because the highest flow rates were not always observed on the final day of sampling, we also analyzed maximum  $Q$  ( $Q_{\text{max}}$ ) during the study. Finally, we investigated maximum induced resin flow ( $Q_{\text{maxinduced}}$ ), the difference between  $Q_{\text{max}}$  and  $Q_{\text{initial}}$ . Welch's  $t$  tests were used to determine differences of  $Q_{\text{final}}$  and  $Q_{\text{max}}$  between attacked and non-attacked trees, as well as  $Q_{\text{maxinduced}}$  for attacked trees. To analyze the success of aggregation pheromones in attracting bark and wood-boring beetles, a general linear model approach was used to test for differences between baited and control trees in the fertilization treatments.

### 3. Results

#### 3.1. Bark beetle response to baiting — experiment 1

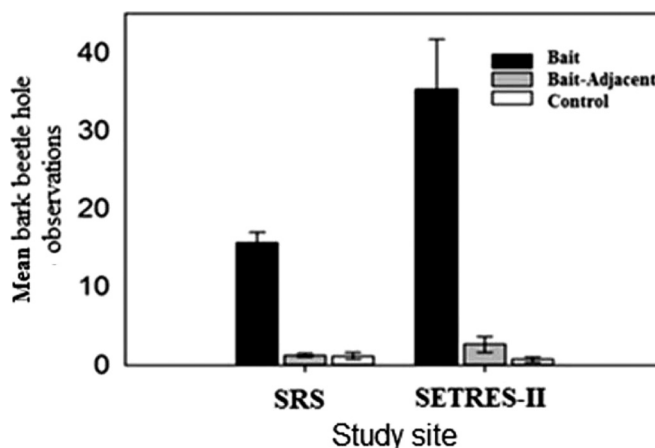
After 1 week since lure installation, between 10 and 25 bark beetle holes were observed on the boles of all baited trees growing in SRS. Two *Monochamus* sp. egg niches were observed on each of two trees. After 21 days, baited trees continued to sustain an increased number of holes (ranging from 6 to 54 additional holes), but no adjacent trees showed any signs of insect damage. After ~70 days, all six baited trees were dead. Within a distance of 5 m from each baited tree, bark beetle holes were observed on between 4 and 11 nearby trees, and mortality (assessed as crown discoloration or lack of needles) was observed for 7 of these neighboring trees on 23 August.

We compared beetle attack intensity at different heights on the stem to determine whether sampling at breast height was representative of the entire tree. Bark beetle holes observed on upper portions of the stem ( $\geq 2$  m;  $A_{\text{high}}$ ) showed a strong linear correlation (Fig. 3;  $R^2 = 0.88$ ,  $P < 0.0001$ ,  $A_{\text{high}} = 1.451A_{\text{low}} + 0.541$ ) with those observed near the base of the stem ( $< 1.4$  m;  $A_{\text{low}}$ ). There were approximately 50% more observed holes on the upper stem than observed near the base of the stem.

#### 3.2. Temporal beetle attack — experiments 2 and 3

During the second experiment occurring at SRS, bark beetle attacks occurred later and at a lower intensity than in the first experiment. Bark beetle holes were observed 25 days after baits were deployed, with 13 of 18 (72%) baited trees attacked by this date. Little change occurred in the number of bark beetle holes until the final sampling date (86 days), at which time 100% of all baited trees and zero non-baited study trees were attacked. Attacked trees were located within 10 m of the bait tree, and bark beetle hole occurrence decreased with increasing distance from the bait (Fig. 2a). We observed sixfold more bark beetle holes on

**Fig. 4.** Bark beetle bark beetle holes observed along the stem of trees sampled for resin flow. Baited trees experienced significantly more holes than adjacent or control trees, highlighting the localized nature of the bark beetles being the primary wounding agent for this study.



baited trees than all others (mean  $\pm$  SE,  $15.6 \pm 1.4$ , compared with  $2.6 \pm 0.7$  and  $2.1 \pm 0.3$  on bait-adjacent and control trees, respectively; Fig. 4). *Ips* species were captured much more frequently on baited trees than on control trees ( $t_{17} = 8.66$ ,  $P < 0.01$ ; Table 2). Total number of *Ips avulsus*, *I. calligraphus*, and *I. grandicollis* were 2567, 72, and 242, respectively (Table 2). We observed BTB pitch tubes on 12 trees (mean  $\pm$  SE,  $3.5 \pm 0.84$ ), of which three trees had more than seven pitch tubes 25 days after bait deployment.

Bark beetle attacks in response to baiting progressed less quickly at SETRES-II than at SRS. Beginning 42 days after baits were deployed (day of year (DOY) 145), bark beetle holes were observed on only 16 trees, and only two trees had more than 3 holes  $\cdot$   $m^{-2}$  (Figs. 5a and 5b). Bark beetle hole counts increased dramatically by DOY 189, when 16 trees had more than 6 holes  $\cdot$   $m^{-2}$ . By the final sampling day (DOY 214), 20 trees had more than 10 holes  $\cdot$   $m^{-2}$ , eight of which had more than 15 holes  $\cdot$   $m^{-2}$ . The number of beetle bark beetle holes on non-baited trees decreased with increasing distance from the bait (Fig. 2b). Attacked trees (with more than 6 holes) were limited to approximately 5 m from the bait. Of the 17 trees that were baited, 88% were considered attacked. Additionally, 29% of the 51 bait-adjacent trees that were monitored experienced bark beetle holes.

Baited trees had over 13 times bark beetle holes than bait-adjacent and control trees (mean  $\pm$  SE,  $35.3 \pm 6.4$ , compared with



**Table 2.** *Ips* spp. sticky trap collections (mean (standard error (SE)) and total number of adults) at the two study sites: SRS (2012) and SETRES-II (2013).

|                            | Tree    | Dates          | No. of adult beetles, mean (SE) |                         |                         |
|----------------------------|---------|----------------|---------------------------------|-------------------------|-------------------------|
|                            |         |                | <i>Ips avulsus</i>              | <i>Ips calligraphus</i> | <i>Ips grandicollis</i> |
| SRS                        | Bait    | 8–14 June      | 36.1 (4.23)                     | 0.5 (0.15)              | 1.53 (0.24)             |
|                            |         | 15–22 June     | 27.2 (3.08)                     | 1.17 (0.98)             | 3.08 (0.35)             |
|                            |         | 22–25 June     | 8.02 (1.39)                     | 0.33 (0.38)             | 2.11 (0.40)             |
|                            | Control | 8–14 June      | 0.03 (0.12)                     | 0 (0)                   | 0 (0)                   |
|                            |         | 15–22 June     | 0 (0)                           | 0 (0)                   | 0 (0)                   |
|                            |         | 22–25 June     | 0 (0)                           | 0 (0)                   | 0 (0)                   |
| SETRES-II                  | Bait    | 7 April–23 May | 3.76 (0.47)                     | 8.29 (0.87)             | 1.76 (0.25)             |
|                            |         | 24 May–7 July  | 7.06 (1.59)                     | 21.41 (3.88)            | 5.82 (1.08)             |
|                            | Control | 7 April–23 May | 0.18 (0.09)                     | 0.29 (0.14)             | 0.06 (0.06)             |
|                            |         | 24 May–7 July  | 0.06 (0.06)                     | 0.53 (0.15)             | 0 (0)                   |
| Total no. of adult beetles |         |                |                                 |                         |                         |
| SRS                        |         |                | 2567                            | 72                      | 242                     |
| SETRES-II                  |         |                | 173                             | 524                     | 129                     |

2.6 ± 0.3 and 0.7 ± 0.4, respectively; Fig. 4). Sticky trap data revealed a significantly higher presence of *Ips* spp. ( $t_{[16]} = 6.60$ ,  $P < 0.01$ ) on baited trees than on control trees. Total number of *Ips avulsus*, *I. calligraphus*, and *I. grandicollis* were 173, 524, and 129, respectively (Table 2). We observed BTB pitch tubes on 23 trees (22.3 ± 4.2); 14 of these had more than 20 pitch tubes 118 days after bait deployment.

### 3.3. Resin flow dynamics

#### 3.3.1. Resin flow response to bark beetle attack

At SRS, attacked trees experienced a significant increase in resin flow over the course of the experiment ( $F_{[1,344]} = 12.97$ ,  $P = 0.0004$ ), indicating that the beetle attack resulted in induced resin flow. Specifically, we observed three times as much resin flow in attacked trees 86 days after bait deployment (DOY 244) than in non-attacked trees (Fig. 5c).

Overall, final resin flow ( $Q_{\text{final}}$ ) was higher in attacked trees than in non-attacked trees, 4.2 g·day<sup>-1</sup> versus 1.3 g·day<sup>-1</sup>, respectively (Fig. 6a;  $t_{[18]} = 4.77$ ,  $P < 0.01$ ).  $Q_{\text{max}}$  was also higher among attacked trees than non-attacked trees, 4.9 g·day<sup>-1</sup> versus 2.3 g·day<sup>-1</sup>, respectively (Fig. 6c;  $t_{[19]} = 4.47$ ,  $P < 0.01$ ). Overall, attacked trees had higher  $Q_{\text{maxinduced}}$  (i.e., a greater difference between  $Q_{\text{initial}}$  and  $Q_{\text{max}}$ ) than non-attacked trees, 3.0 g·day<sup>-1</sup> versus 0.2 g·day<sup>-1</sup>, respectively (Fig. 6e;  $t_{[18]} = 4.75$ ,  $P < 0.01$ ).

At SETRES-II, attacked trees experienced a significant increase in resin flow over the course of the experiment ( $F_{[1,324]} = 19.13$ ,  $P < 0.0001$ ). Resin flow of non-attacked trees remained low (<~1 g·day<sup>-1</sup>) throughout the experiment. In contrast, resin flow was considerably elevated in attacked trees (Fig. 5d).  $Q_{\text{final}}$  was higher in attacked trees than in non-attacked trees, 2.3 g·day<sup>-1</sup> versus 1.6 g·day<sup>-1</sup>, respectively (Fig. 6b;  $t_{[21]} = 3.02$ ,  $P < 0.01$ ).  $Q_{\text{max}}$  was also higher among attacked trees compared with non-attacked trees, 3.2 g·day<sup>-1</sup> versus 0.6 g·day<sup>-1</sup>, respectively; as was  $Q_{\text{maxinduced}}$ , 1.9 g·day<sup>-1</sup> versus 0.3 g·day<sup>-1</sup>, respectively (Fig. 6d,  $t_{[21]} = 3.0$ , and Fig. 6f;  $t_{[21]} = 2.59$ ,  $P < 0.01$ , respectively).

#### 3.3.2. Fertilization effects on resin flow

At SRS,  $Q_{\text{initial}}$  was higher in fertilized trees than in non-fertilized trees ( $t_{[86]} = 1.99$ ,  $P < 0.05$ ), but there was no difference at SETRES-II ( $t_{[80]} = 1.99$ ,  $P = 0.51$ ). At SRS, fertilization had no effect on  $Q_{\text{final}}$  or  $Q_{\text{max}}$  in non-attacked trees (Figs. 6a and 6c). Within fertilized plots, attacked trees had significantly higher  $Q_{\text{final}}$  than non-attacked fertilized trees ( $t_{[7]} = 2.51$ ,  $P = 0.03$ ); however, no significant fertilization treatment effect was observed when comparing  $Q_{\text{final}}$  between fertilized and unfertilized plots ( $t_{[15]} = 0.91$ ,

$P = 0.38$ ). Attacked non-fertilized trees experienced increased resin flow one sampling period earlier (approximately 21 days) than fertilized trees, despite similar timing of beetle attack (Figs. 5a and 5c). Fertilization had no effect on  $Q_{\text{max}}$  of non-attacked ( $t_{[68]} = 0.61$ ,  $P = 0.54$ , and  $t_{[70]} = 1.38$ ,  $P = 0.17$ , respectively) or attacked trees ( $t_{[15]} = 0.58$ ,  $P = 0.57$ , and  $t_{15} = 1.30$ ,  $P = 0.22$ , respectively). Similarly, we observed no fertilization effect on  $Q_{\text{maxinduced}}$  of attacked trees ( $P > 0.05$ ).

Again, at SETRES-II, fertilization had no effect on  $Q_{\text{final}}$  or  $Q_{\text{max}}$  in non-attacked trees ( $t_{[47]} = 0.68$ ,  $P = 0.50$ , and  $t_{[47]} = 1.11$ ,  $P = 0.27$ , respectively).  $Q_{\text{final}}$  was higher in attacked fertilized trees, although results were not significant ( $t_{[17]} = 1.09$ ,  $P = 0.29$ ). Fertilization had no effect on  $Q_{\text{max}}$  in attacked trees ( $t_{[17]} = 0.52$ ,  $P = 0.61$ ).

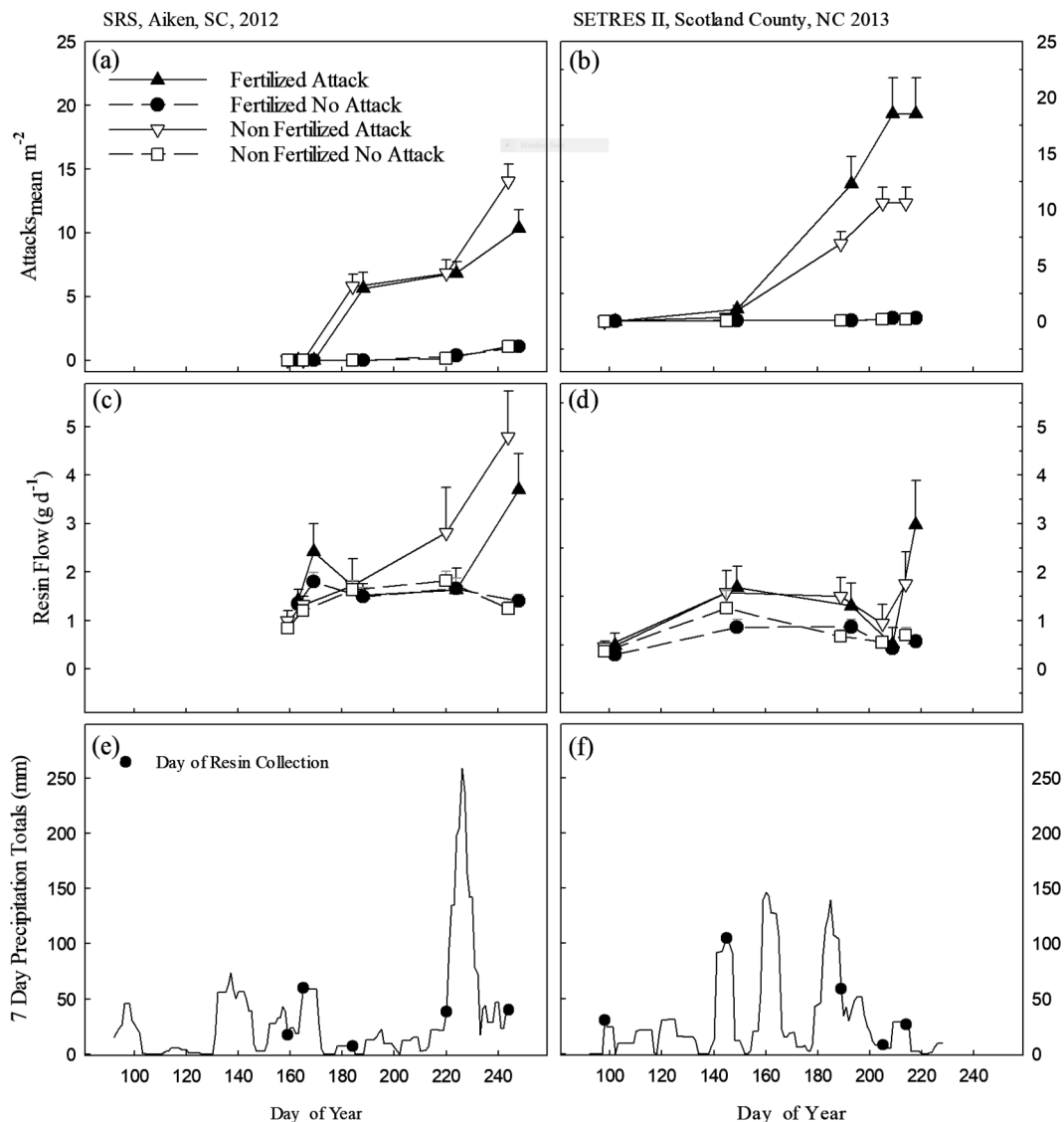
## 4. Discussion

### 4.1. Effects of synthetic pheromone lures on bark beetle attacks

Our research objective was to determine whether using baits to create localized bark beetle attacks could facilitate mechanistic studies of induced pine tree resin defenses. Both our pilot study and replicated studies support previous successes for using aggregation pheromones as a method to attract bark and wood-boring insects into an area of interest and promote beetle attack. Results from the pilot study demonstrate that this technique was capable of producing localized pockets of mortality in baited trees and their neighbors. Mortality did not occur in the replicated studies. Importantly, however, an induced resin flow response was observed in both study sites. The spatial extent of herbivory in response to the baiting was limited to target trees and adjacent trees, with trees greater than 10 m from the bait showing no evidence of increased beetle activity (Fig. 2).

Bark beetle attack was achieved quickly after aggregation pheromone installation. While bark beetle hole abundance was not particularly high when compared with previous experimental baiting studies (Schlyter et al. 1987; Schlyter and Anderbrant 1989), the level of attack was sufficient to produce an increase in resin flow. It should be recognized that different species of *Ips* beetles colonize different sections of the host tree (Paine et al. 1981); however, a high number of *I. avulsus* were identified on sticky traps even though this species tends to occupy the upper stem and crown (Connor and Wilkinson 1983). We observed a strong linear correlation between holes on the lower stem and upper stem, suggesting that total beetle activity affecting the stem would be greater than what was observed on the ground, especially considering that some species preferentially attack the crown.

Fig. 5. Mean bark beetle attack (number·m<sup>-2</sup>) at (a) SRS and (b) SETRES-II; mean resin flow by site treatment for non-attacked and attacked trees at (c) SRS and (d) SETRES-II; total of prior 7-day precipitation totals for (e) SRS and (f) SETRES-II.



We emphasize that in this study, the primary goal was to see if baits could produce an induced resin flow response over the course of a growing season. The sampling plan did not include long-term monitoring to determine the extent of colonization or sustained populations but investigating the potential for this approach to produce long-term changes in beetle populations and resin production is a good avenue for future research.

#### 4.2. Resin flow response

In the replicated experiments at SRS and SETRES-II, an induced resin flow response was observed for the attacked trees. At both sites, we observed that resin flow increased in trees after they were attacked and that the attacked trees produced consistently more resin than their non-attacked neighbors (Figs. 5c, 5d, and 6). No difference between initial and final resin flow was observed in non-attacked trees (Figs. 5c and 5d), suggesting that higher final resin flow in attacked trees was in response to the beetle attack and not seasonal variation or ancillary effects of mechanical wounding. The spike in resin flow of attacked trees at the end of the growing season in both SRS and SETRES-II (Figs. 5c and 5d) suggests an induced response of newly synthesized resins to continue defense during the bark beetle attack (Lombardero et al.

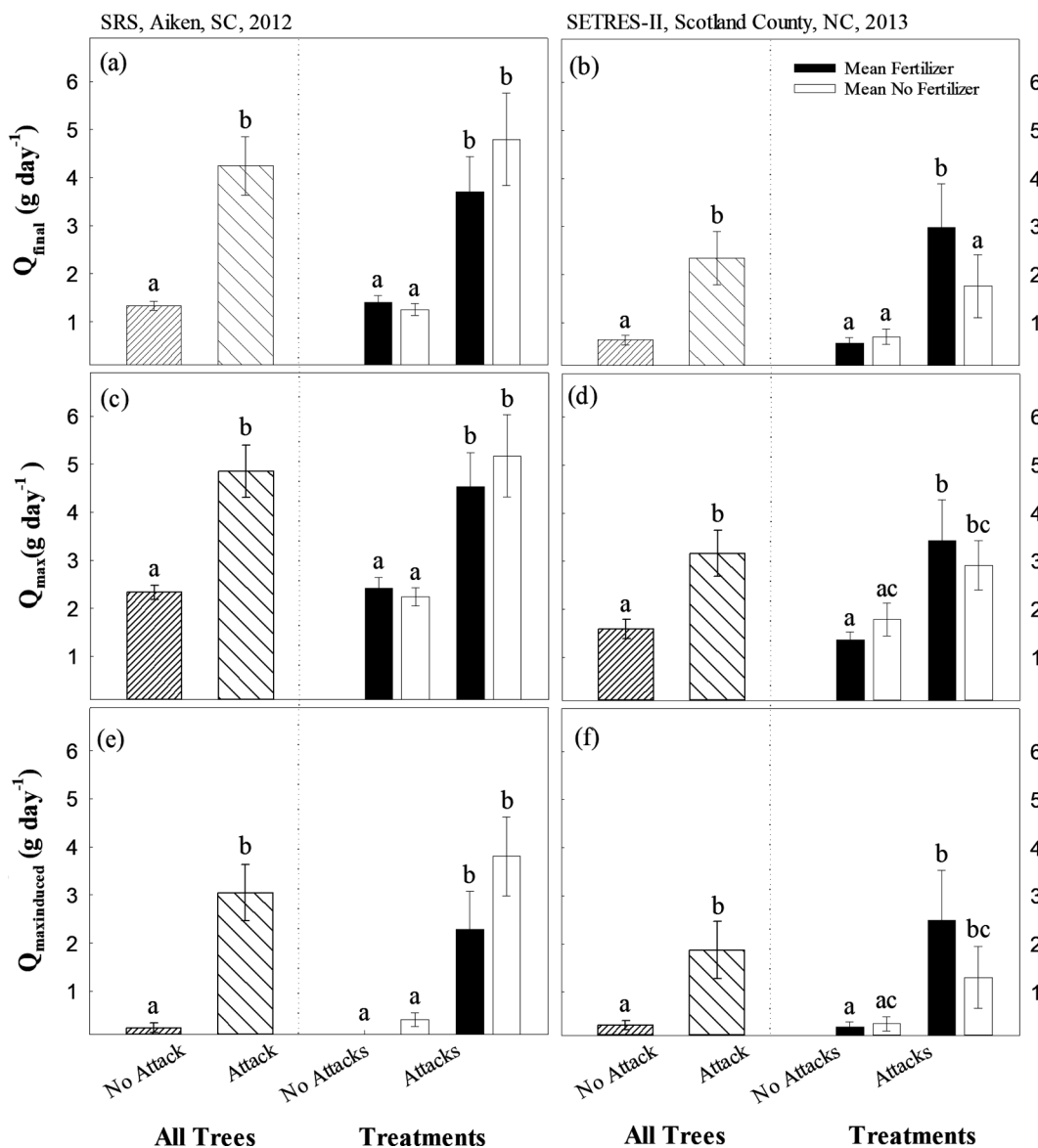
2000). Because much of what is known about resin flow dynamics concerns constitutive flow rates only (Hodges et al. 1977; Warren et al. 1999; Gaylord et al. 2007), the approach described here has the potential to enhance our understanding of the mechanisms of resin flow production before and during bark beetle herbivory. Here, we limited the scope of our analysis to focus on the physical aspect of resin defense, which is an important but ultimately incomplete description of the integrated defense system of pine trees. Future work could use this approach with a greater emphasis on the chemical composition of resin exudates, in terms of both constitutive and induced resin chemical composition. Furthermore, by modifying this approach to target specific insects with different types of bait, future research could help to improve our understanding of how tree defense responses may depend on the attacking species.

##### 4.2.1. Fertilization effects on resin flow

The observed effects of fertilization on resin flow were subtle and differed among sites. At SRS, the temporal pattern of beetle arrival was nearly identical among fertilization treatments until the final sampling date, when more bark beetle holes were observed in non-fertilized trees. Similarly, at SETRES-II, fertilized



**Fig. 6.**  $Q_{\text{final}}$  at (a) SRS and (b) SETRES-II;  $Q_{\text{max}}$  at (c) SRS and (d) SETRES-II;  $Q_{\text{maxinduced}}$  at (e) SRS and (f) SETRES-II. All trees non-attacked (fine cross-hatching) and attacked (thick cross-hatching). (Different lowercase letters represent significant differences at  $\alpha < 0.05$ .)



and non-fertilized trees followed a similar pattern of bark beetle arrival for the first two sampling events; however, unlike SRS, once bark beetle arrival passed our metric for threshold of attack, fertilized trees showed consistently higher bark beetle hole counts than non-fertilized trees.

Within the scope of our experiment, our results only partially support the growth-differentiation balance hypothesis (GDBH). In contrast to the GDBH, but consistent with previous results observed by Novick et al. (2012), Klepzig et al. (2005), vanAkker et al. (2004), and Lombardero et al. (2000), we found no consistent significant effect of fertilization treatments on constitutive resin flow at SETRES-II. However, at SRS, fertilized trees produced ~60% higher initial flow than non-fertilized trees, in contrast to results presented by Warren et al. (1999) reporting that fertilized trees produced as much as 50% lower resin flow than non-fertilized trees. The results from SRS are inconsistent with predictions from the GDBH, which states that when conditions (such as nutrient availability) are favorable for growth, carbohydrates are preferentially allocated for growth processes rather than defense (Herms and Mattson 1992). We would expect that initial flow would be

greater for non-fertilized trees compared with fertilized trees, which is not what we observed at SRS and suggests that growth was limited by something other than nutrients (i.e., energy) allowing the fertilized trees to allocate resources to secondary processes. No significant fertilizer effect was observed for SETRES-II.

Research from Moreira et al. (2015) suggests that trade-offs between growth and defense are determined not only by resource availability (i.e., nutrients), but also by genetics and genetic-nutrient interactions. Sampedro et al. (2010) demonstrate that phenotypic variation in leaf volatile terpene concentrations is affected by genetic variation and not nutrient (in this case, phosphorus) availability in certain pines. This could explain why the results from SRS and SETRES-II were different, with fertilized trees exuding more initial resin and less final resin than non-fertilized trees at SRS and no difference in initial resin flow and higher final resin flow at SETRES-II. Further, in non-attacked trees at both SRS and SETRES-II, we reported no differences in resin flow throughout the experiment between fertilized and non-fertilized trees, suggesting that fertilization has no effect on constitutive flow.

## 5. Conclusion

We modified the utility of synthetic pheromone lures to attract bark beetles into a localized pocket of attack to elicit a resin flow defense response without the threat of a major outbreak. Attack levels were sufficient to cause mortality in the first experiment; in the subsequent resin flow experiments, attack levels were not sufficient to cause mortality but were clearly sufficient to elicit an increased resin response. We achieved increased resin flow rates in attacked trees and confidently attribute these increased resin flow rates to bark beetle invasion. Future studies may utilize this method to further investigate environmental and management effects on inducible physiological defenses in conifer trees. With this approach, we are better equipped to design experiments investigating invading bark beetle – host tree defense responses and the factors or disturbances (i.e., fire, water stress) that may exacerbate or hinder this response.

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