Fine-scale features on bioreplicated decoys of the emerald ash borer provide necessary visual verisimilitude

Michael J. Domingue[a], Drew P. Pulsifer[b], Mahesh S. Narkhede[c], Leland G. Engel[d], Raúl J. Martín-Palma[e], Jayant Kumar[c], Thomas C. Baker[a], and Akhlesh Lakhtakia[b,*]

[a]The Pennsylvania State University, Department of Entomology, University Park, PA 16802, USA
[b]The Pennsylvania State University, Department of Engineering Science and Mechanics, University Park, PA 16802, USA
[c]University of Massachusetts Lowell, Center for Advanced Materials and Department of Physics and Applied Physics, Lowell, MA 01854, USA
[d]The Pennsylvania State University, Department of Mechanical and Nuclear Engineering, University Park, PA 16802, USA
[e]Universidad Autónoma de Madrid, Departamento de Física Aplicada, 28049 Cantoblanco, Madrid, Spain

ABSTRACT

The emerald ash borer (EAB), *Agrilus planipennis*, is an invasive tree-killing pest in North America. Like other buprestid beetles, it has an iridescent coloring, produced by a periodically layered cuticle whose reflectance peaks at 540 nm wavelength. The males perform a visually mediated ritualistic mating flight directly onto females poised on sunlit leaves. We attempted to evoke this behavior using artificial visual decoys of three types. To fabricate decoys of the first type, a polymer sheet coated with a Bragg-stack reflector was loosely stamped by a bioreplicating die. For decoys of the second type, a polymer sheet coated with a Bragg-stack reflector was heavily stamped by the same die and then painted green. Every decoy of these two types had an underlying black absorber layer. Decoys of the third type were produced by a rapid prototyping machine and painted green. Fine-scale features were absent on the third type. Experiments were performed in an American ash forest infested with EAB, and a European oak forest home to a similar pest, the two-spotted oak borer (TSOB), *Agrilus biguttatus*. When pinned to leaves, dead EAB females, dead TSOB females, and bioreplicated decoys of both types often evoked the complete ritualized flight behavior. Males also initiated approaches to the rapidly prototyped decoy, but would divert elsewhere without making contact. The attraction of the bioreplicated decoys was also demonstrated by providing a high dc voltage across the decoys that stunned and killed approaching beetles. Thus, true bioreplication with fine-scale features is necessary to fully evoke ritualized visual responses in insects, and provides an opportunity for developing insect-trapping technologies.

Keyword: 3D Printing, Bioreplication, Bragg-stack reflector, Engineered biomimicry, Structural color, Spectral emission, Stamping

1. INTRODUCTION

1.1 Buprestid biology

An eruptive outbreak of the emerald ash borer (EAB), *Agrilus planipennis*, in North America over the past decade has resulted in a serious threat to urban and forest ecosystems,[1] with tens of millions of ash trees destroyed. This outbreak has highlighted the overall potential threat of buprestid beetles (Coleoptera: Buprestidae), a large and

[*] Corresponding author. E-mail: akhlesh@psu.edu

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diverse family of insects, estimated to include 15,000 species.[2] Actual and potential impacts of these insects range from accidental introductions associated with travel and trade, to shifts in host susceptibility induced by climate change.

Buprestids are typically associated with trees and shrubs, upon which eggs are laid in bark crevices. Larvae then tunnel into the inner bark which can result in host mortality if the damage becomes severe. In many cases, the adults can be found intermittently feeding and mating on the foliage of associated host plants, although little damage to the plant occurs at the adult stage.

Adult buprestids are conspicuous by their bright, metallic coloring, which has long been implicated as a mechanism for mate attraction. Tree-feeding buprestids such as EAB are known to rest and feed on leaves as adults. Adult males patrol the canopy for females, alighting onto them after direct flights from 30-100 cm above. Dead EAB beetles pinned on ash leaves as decoys are known to repeatedly evoke such male flights directly onto the decoys.[3] The term *paratrooper copulation* was used to describe this ritualistic behavior, as it was often immediately followed by vigorous attempts to copulate with the decoy.

1.2 Necessity of monitoring tools

The emergence of *A. planipennis* as a severe pest in North America has led to a renewed interest in research into the behavior and ecology of buprestids with the goal of developing methods for early detection of their infestations. Early detection of incipient infestations of healthy forests is crucial to maximize management options. Currently, large purple or green *prism* traps covered in transparent sticky glue are used to trap the beetles. These traps require a high amount of labor, as (i) they must be deployed using ropes and (ii) beetles must be removed from the sticky glue along with a large number of untargeted insects which share a similar level of attraction to such traps.

Research into the ritualistic paratrooper-copulation behavior has also been applied to develop an alternative trapping approach. This exploitation of male mate searching allows us to create a smaller and more efficient trap that can also detect EAB in an infested area with a high level of success.[4] By simply pinning real dead EAB females onto leaves or small green plastic surfaces, which are covered with transparent glue, males beetles can be ensnared as they approach and try to mate with the decoys.[4,5,6] However, it is impractical to hope to deploy natural materials such as real decoy beetles in a large-scale trapping program because the beetles degrade, breaking into fragments as time goes by. Furthermore, the sticky glue that is applied to them appears to darken their color, which may also reduce their effectiveness if they no longer appear similar to live resting females. The use of artificial visual decoys will hopefully bypass these difficulties. In addition to the problem of using natural materials, it is also desirable to develop trapping technologies that do not rely on sticky glue for retention of insects for later identification and assessment of forest health threats. The specific point-source attraction of the male buprestid beetles to the decoys should also allow for novel detection mechanisms that allow real time reporting of the presence of these pests.

1.3 Development of usable artificially fabricated decoys

In this paper, we describe two different modes for the production of artificial decoys and their application for evoking behavioral responses from other beetles and allowing captures of real beetles *in situ*, both with and without sticky material. Artificial decoys of two types employed by us were fabricated using a set of two dies cast from an actual EAB specimen. The industrially scalable bioreplication technique employed produced decoy with fine-scale features, including pits and spicules, found on the elytra of EAB. Artificial decoys of the third type were manufactured by 3-dimensional (3D) printing and did not have the fine-scale features.

Artificial decoys of all three types were presented to wild populations of males in three different assays, in order to determine which features of the beetle are required to make an adequate decoy that fully evokes the male mating behavior, and how such features can be used to manipulate behavior for a variety of trapping applications. The first assay involved simply pinning the artificial decoys alongside real beetles on leaves and observing the frequencies of male approaches and attempts to mate with the various types of decoys. The second assay involved presentation of the decoys on stick *branch traps* with green plastic surfaces that mimic leaves, which will ensnare any male beetles attempting to mate with the decoys. The final assay involved an electrocution trap, whereby a voltage sufficient to stun or kill a beetle is applied to the decoys using two steel pins. Approaching beetles will be stunned if they attempt to mate with the decoys, after which they will be collected in a cup below.

2. METHODOLOGY

2.1 Field sites

Trapping and observation of wild EAB occurred at a site directly on the Pennsylvania State University campus in University Park, PA, USA (40° 48' 40"N, 77° 50' 41W, 318 m altitude). The dominant species at this site of approximately 2000 trees was green ash (*Fraxinus pennsylvanica*). The trees were uniformly planted 35 years ago in rows that are four meters apart. Throughout this site there were strong signs of EAB infestation, which is easily observable from trees that are either completely dead, or fail to produce leaves in the crown. Often such trees have epicormic sprouting of new branches and leaves lower on the trunk.

The site at which we have performed all trapping experiments and observations of TSOB has been at an oak forest near Mátrafüred, Hungary (47°50'17"N, 19°59'50"E, 367 m altitude). Active logging occurs regularly at this site and log piles that are attractive to buprestids are often left along the road side for prolonged periods. The composition of this forest is dominated by sessile oak, *Quercus petraea*. This site has been also been used for investigations into the behavior of and ability to trap other oak buprestid species, such as *Agrilus sulcicollis*, and *Agrilus angustulus* from 2009 to 2013.[6,7]

2.2 Natural decoys

To obtain real EAB decoy to compare to our fabricated ones, we relied on the USDA-APHIS EAB rearing facility in Brighton, MI, USA. A colony is maintained year round at this facility using cut logs of *F. pennsylvanica*. For the sake of uniformity, only females were used for experiments, although EAB males will also evoke a response from other males. The females used for experiments were collected 30 days before being used in our trapping and observational experiments. They were collected as they emerged as adults from the colony and killed by placing them in a freezer (-20°C) for approximately 48 hours. Right after removing them from the freezer they were pinned through the prothorax so they could be affixed to leaves and traps for our experiments. Previously, it had been shown that real EAB decoys prepared in this manner were attractive to EAB males[5], as well as to TSOB males and males of other European oak buprestids.[6] Six TSOB female specimens that had been hand collected in previous years were also saved and pinned for use in observation experiments.

2.3 Bioreplicated decoys

Bioreplicated decoys of two types were produced as described in detail elsewhere.[8] Briefly, for a realistic duplication of the cuticle surface, a Bragg-stack reflector comprising alternating layers of polyvinyl cinnamate and polyacrylic acid was spin-coated on one side of a polyethylene terephthalate (PET) sheet so that the PET sheet acquired a green color on reflection. The other side of the PET sheet was coated by black absorber paint.

A negative die of nickel was fabricated directly from a dead EAB female, by using an industrially scalable bioreplication technique. From this negative die, a complementary positive die of epoxy was fabricated. Together, the two dies constituted a mold. The PET sheet was lightly molded to produce the bioreplicated decoys, labeled as Biorep1 throughout the text. Bioreplicated decoys labeled as Biorep2 were produced by heavy molding (which destroyed the Bragg-reflector stack) and subsequent painting by a metallic green spray paint.

2.4 3D-printed decoys

We also fabricated decoys of a third type by a less time-intensive method, without attempting to preserve the fine-scale features of the elytra. First, the sagittal profile of a dead EAB female with its elytra folded over its abdomen in resting position was used to devise a 3D model that has roughly the same proportions as a real EAB female. Then, a 3D printer was used to print 300 decoys in 11 discrete 0.254-mm-thick layers of white acrylonitrile butadiene styrene (ABS). The decoys produced in this manner have a stepped surface structure as a result of the printing process. To ensure that this feature did not negatively affect decoy performance, one half of the decoys were smoothed by rubbing them with acetone which dissolves the ABS. Regardless of this treatment all decoys were painted with a metallic green spray paint. An awl was used to pierce a hole through the decoy at approximately the corresponding position as used in the real beetles. This hole was used for pinning the beetles to the traps. Subsequent testing revealed no effect of the smoothing effect applied to half the decoys, and this feature will not be discussed any further.

2.5 Observational experiment

Using standard protocols developed for the species,[7] TSOB behavior was observed at the Mátrafüred, Hungary site in response to real and fabricated decoys. First we located south-facing low-lying branches of sessile oak trees, where insects of this species could be seen flying and occasionally resting at a visible height of approximately 2 m. At each observation period, the following specimens were pinned to neighboring leaves: (1) a real female EAB, (2) a real female TSOB, (3) Biorep1 decoy, (4) Biorep2 decoy, and (5) a 3D-printed decoy. The leaves selected were approximately 10 cm apart, and observed for 10-min periods. After each period the decoys were re-arranged randomly, such that each decoy was now placed on a new leaf. Observations continued as such for 10-min periods until the beetles were no longer active on a given day. Several behaviors were observed including, (1) initial flight in the direction of a decoy from about 1 m away, (2) how often these approaches led to the male landing on the decoy, and (3) finally the duration of time spent on the decoy and whether or not copulation was attempted. Observations were made daily from June 10 to 19, 2013 generally during any active periods between 1130 and 1500 hours when the weather was favorable. Similar observation experiments are planned for the EAB in 2014.

2.6 Sticky traps with decoys

Traps were deployed at the EAB field site, which were identical to those previously used for TSOB in Hungary for other studies.[6,8] These traps consisted of glossy white weatherproof cardboard structures fitted with green plastic surfaces to mimic leaves. The white cardboard portion consisted of two upper 5×9 cm^2 surfaces and two lower 9×13 cm^2 surfaces forming a continuous structure with an internal gap that could be used to insert tree branches to hang the trap. The upper surfaces were covered by green corrugated plastic cards of the same size and were oriented toward the sun at approximately 45°. The green plastic was measured to have a peak reflectance at 540 nm. For placing the traps, we located sunlit clearings, which also had south facing branches where the traps could be hung. This maximized the amount of time when the traps, and the decoys placed on them, would be illuminated by sunlight. Traps were placed within standing reach of an average adult. Thus they tended to range from 1.5-2.5 m from the ground.

The decoys were not added to the traps until they had been secured to the branches. If a trap was to have dead female *A. planipennis* or fabricated decoys affixed, the desired decoys were pinned to the center of both of the two upper green plastic surfaces of the trap. The upper surfaces of the traps were also bounded with 2×9 cm^2 glossy cardboard strips to prevent the beetles from falling off the sloped trap surface onto the ground. Next, Tanglefoot™, a sticky clear glue, was applied over the entire horizontal surface of the branch-traps. A very thin layer also covered the decoy itself to ensnare any beetles directly landing on it as was previously shown to be effective.[4,5,6] In a previously described experiment, bioreplicated decoys were attached to these traps and compared to those fitted with real beetles for catching TSOB in Hungary.[8]

In the summer of 2013 at the Pennsylvania site, an experiment was performed to assess the possible use of 3D printed decoys on these traps. The experimental design included three traps per tree, each with one of these treatments: (1) a control trap, (2) with real EAB females, and (3) with 3D- printed decoys. A dispenser of the compound (Z)-3-hexen-1-ol, which is emitted by many types of leaves, was added to each tree to further attract beetles to the area where they could search for the decoys. There were 22 replicates of each treatment deployed. Traps were checked on each day of the experiment and all insects or plant materials ensnared on the sticky material were removed. All the buprestids including EAB were retained in plastic bags for later identification. Similar experiments were performed for TSOB in June 2013, but the data have not yet been analyzed.

2.7 Electrocution traps with bioreplicated decoys

The main body of the trap was a 0.5 m long x 10-cm-diameter polyvinyl chloride (PVC) pipe. The top of the pipe was fitted with a funnel and a flange for hanging the trap. Above the opening to the funnel a platform was constructed with two 9 × 13 cm^2 green plastic cards oriented toward the sun at 45°, and also positioned so that beetles could fall from one of the plastic squares into the funnel and trap body after being electrocuted. The cards were identical to those used for the sticky branch traps. Only artificial decoys were used in these traps. Real beetles are not electrically conductive after becoming dry and are also easily broken apart in such a preparation. Additionally, because of the evidence that 3D-decoys did not provide a signal that drew males to touch them (Section 3.1), they also were not used.

To make an electrified decoy, the bioreplicated decoy was affixed to the central surface of the card with a steel pin in a position directly over the funnel. A wire fitted with an alligator clip was attached to this pin so that it could be removed and the decoy replaced whenever desired. Another steel pin was permanently positioned just below the decoy and soldered permanently to a wire. The wires attached to the pins were connected to a transformer that provided a 4-kV potential. The transformer had been removed from a commercially available battery-operated electric fly swatter and attached inside the main body of the trap.

For the first stage of deployment, from June 8 to 24, 2013, four of the traps were run at the Hungarian oak forest where all work on TSOB has been performed, while three were also run at the Pennsylvania site for EAB. After terminating work in Hungary and repairing some of the traps that had been damaged by strong rain storms, all seven traps were run from July 1 to 11, 2103 at the Pennsylvania site. Roughly half of the lures were Biorep1 versus Biorep2, with an extra Biorep2 always being deployed at the Pennsylvania site, which had an odd number of traps. The same (Z)-3-hexen-1-ol dispensers described above were added to the traps to enhance attraction. Traps were only activated during dry periods in the daytime. Otherwise, rain and morning dew short the electric circuit and quickly discharge the batteries completely.

3. RESULTS

3.1 Observational experiment

We observed male TSOB flying toward all of the decoys with the following frequencies EAB (32), TSOB (23) Biorep1 (21) Biorep2 (18) and 3D-printed (15). Those numbers include all initial approaches, regardless of whether the flight was complete and the beetles landed on the decoy targets. When flying toward the real beetles, nearly all the approaching TSOB males landed on the decoys (96%), with many subsequently trying to copulate with the dead beetle (27%). A large percentage of those flying toward the bioreplicated decoys actually alighted directly on them (74%), but they never remained on the decoys and attempted to copulate them. For the 3D-printed decoys, which do not bear a replication of the fine surface structure of the cuticle, the wild beetles almost never landed on the decoys (7%) and never tried to copulate. Whenever beetles did not alight on the decoy they would instead land on neighboring leaves or fly away toward other leaf clusters

3.2 Sticky traps with decoys

For the entire season, there were 8 males and 8 females on the blank traps, 89 males and 25 females on the EAB-baited traps, and 84 males and 23 females on the 3D-printed decoy-baited traps. EAB were caught at all of the 22 trees where traps were deployed except for one tree, which was located on the far western end of the plot away from the infested area. The traps at this location were also shaded for much of the day by other surrounding *Fraxinus* and *Quercus* trees, with only one or two hours of direct sunlight reaching the traps each day.

While the data are not completely tabulated for the parallel experiment with TSOB in Hungary, it should be noted that dozens of TSOB were also caught on these traps. However, there were almost certainly relatively more captures on the blank traps in Hungary. This phenomenon has occurred in other experiments in Hungary and is perhaps related to the large number of small buprestids that exist in such oak forests. These ubiquitous creatures will cover both blank and decoy baited traps. Once a few have landed on a blank trap, they may themselves become decoys for other buprestids including TSOB. In the ash forest in Pennsylvania, there were very few other buprestids caught on any of the other traps, so this is not a concern.

3.3 Electrocution traps with bioreplicated decoys

Over the course of the season 16 EAB and 4 TSOB were caught in the electrocution traps. Of these, 80 percent of the captures were male, including one EAB male that was directly observed flying onto the decoy, becoming electrocuted, and falling into the trap. There were also 65 other smaller oak buprestids in addition to TSOB caught in Hungary, which were primarily *A. angustulus* and *A. obscuricollis*. The only other non-target insects caught included a handful of bright metallic scarab beetles and some hoverflies (Syrphidae), both in Hungary. The numbers of catches for any of the target or non-target insects were not affected by whether Biorep1 versus Biorep2 was used.

3. CONCLUSIONS AND FUTURE DIRECTIONS

While the data concern the behavior of two different species, EAB and TSOB, to the artificial decoys, some general inferences can be made about their efficacy for trapping. Any of the decoys would cause some positive behavioral response by TSOB males, but the fine-scale structural features of the real beetles or bioreplicated decoys were necessary to cause the beetles to touch the decoys. Thus the 3D-printed decoys may not be as effective if they are deployed in particular trapping technologies, such as the electrocution traps, which require the wild beetles to actually touch the decoys. However, they may work perfectly well for traps such as the sticky traps, for which beetles simply need to land within a few cm of the decoy to become trapped. This was proven true for the experiment deploying such decoys for EAB on sticky traps on ash trees, which were as effective as traps baited with real beetle decoys.

Another important finding is that there were never any differences in behavioral responses with respect to the type of artificial decoy used. This has implications for the potential manufacturing of such decoys, because applying green paint to the decoys is much cheaper and easier to consistently perform in comparison to stamping the Bragg Stack layer on top of the beetle dye. The color emissions of the materials differ primarily in the emission of color in very far red to infrared wavelength, which are not likely to be seen by the insects. However, many green pigments, including those found in leaves, do have reflectance at slightly lower wavelengths in the red to far red range, which the real beetle cuticle and fabricated Bragg-stack reflector both lack and could conceivably inhibit the male mating flights. Further research is needed to assess the sensitivity of the approaching male beetles to such aspects of the spectral emission profile. However, it can currently be assumed that for future application any paint used on decoys should be similar to that used for these experiments.

The electrocution traps provide an opportunity to develop technologies that will vastly improve the labor costs and responsiveness of government agencies and private entities who wish to monitor ash forests for the presence of this deadly pest. Much time is required to deploy the industry-standard large sticky prism traps, which must be hung from ropes, and beetles individually picked out of the sticky glue. Furthermore chemical treatment is required to clear the glue from the beetles for identification. The electrocution traps, which have now been proven as conceptually viable alternatives, now need to be refined to increase their usability. For example, shorting events from rain and dew prevented their long-term deployment and thus required us to replace the batteries daily. A solar power supply can alleviate this problem. Additionally, while these traps are fairly small, they need to be further miniaturized and constructed with lighter material.

Another important future direction for trap development is the adaptation of these visual decoy-based systems to remote detection networks. Positive events, such as the electrocution of beetles that touch the decoys, could be reported via satellite to allow real-time pest detection. Further research into eliminating the reporting of false positive events is required to make such a technology viable. This might be accomplished by developing graded electrical delivery systems that narrow the range of insect sizes that will be affected by the current. Additionally, other detection mechanisms may be developed to assess and report other identifiable characteristics of the approaching insects.

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