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Developing a Biological Control Program for the Invasive Goldspotted Oak Borer (*Agrilus auroguttatus* Schaeffer) in Southern California

A Dissertation submitted in partial satisfaction
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by

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DEDICATION

This dissertation is dedicated to my family. Thank you for all the love and support!

To Allison, Mia, and Emma:

You can accomplish anything with hard work and determination. Always remember that life is what you make it…you rule your destiny☺ Auntie Nessa loves you!

To Mom and Dad:

Thanks for always believing in me and giving me the confidence to follow my dreams. I love you both so much!

To my husband Billy:

Your never-ending support and encouragement made the toughest days bearable, and the not-so tough days beautiful. Thank you for always making life easier☺ I love you!

ABSTRACT OF THE DISSERTATION

Developing a Biological Control Program for the Invasive Goldspotted Oak Borer (*Agrilus auroguttatus* Schaeffer) in Southern California

by

Vanessa Marie Lopez

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, August 2013
Dr. Mark S. Hoddle, Chairperson

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, (Coleoptera: Buprestidae) is an invasive wood-boring beetle that aggressively attacks native oak trees in southern California, U.S.A. Native to Arizona, this beetle was initially detected in the Cleveland National Forest, San Diego County, California in 2004, but was likely introduced accidentally several years earlier through movement of infested oak firewood. Prior to its introduction and subsequent invasiveness in southern California, *A. auroguttatus* was rarely collected and very little information was known about this insect. The continuing ecological and economical damage caused by *A. auroguttatus* in southern California has made the development of a classical biological control program for this invasive pest a high priority. However, the implementation of an effective classical biological control program for any invasive species requires the knowledge of several critical components such as the pest's biology, area of origin, natural enemies (in the home and introduced range), and life history traits. Consequently, in order to acquire the basic information needed to initiate a classical biological control program for *A. auroguttatus* in California,

this dissertation research had the following four objectives: 1) determine the fecundity and longevity of *A. auroguttatus* under varying diet and mating treatments, 2) assess the dispersal capabilities of *A. auroguttatus* adults in the laboratory using computerized flight mills, 3) use molecular methods to identify the area of origin for the California population of *A. auroguttatus*, and 4) survey for natural enemies of this beetle by deploying *A. auroguttatus* egg masses into the native and introduced range. Findings of this work showed that a carbohydrate-enriched diet increased longevity and fecundity, and the nutritional status and body size of *A. auroguttatus* adults had a significant influence on overall flight performance. The area of origin was not determined conclusively, although data suggests the Dragoon Mountains in Cochise Co., Arizona as a possible source for the California population of *A. auroguttatus*. Additionally, the first known egg parasitoid of *A. auroguttatus* was collected in AZ, and identified as *Trichogramma* sp. using molecular techniques. The results of this dissertation research will further the management of *A. auroguttatus* in southern California.

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Chapter 1

Goldspotted Oak Borer: An Introduction into the History, Biology, and Management of an Important Oak Pest in Southern California

History of Goldspotted Oak Borer

Taxonomy. The name goldspotted oak borer (Coleoptera: Buprestidae) is used to describe two species of phloem/wood-boring beetles, *Agrilus auroguttatus* Schaeffer and *Agrilus coxalis* Waterhouse. These two species are nearly identical in appearance, but have different historical distribution records. *Agrilus coxalis* was originally described in 1889, and a female specimen collected in Juquila, Mexico was designated as the lectotype (Waterhouse 1889). Since its description, specimens of *A. coxalis* have been collected throughout southern Mexico (i.e., in the states of Cordova, Chiapas, Veracruz, Jalisco, and Oaxaca), and also in northeastern Mexico and Guatemala (Coleman and Seybold 2011). Schaeffer (1905), first described *A. auroguttatus*, and representative specimens of this species have been collected primarily in Arizona, although one specimen was collected from Baja California Sur, Mexico in 1977 (Coleman and Seybold 2011). Hespenheide (1979) synonymized *A. auroguttatus* with *A. coxalis* since their morphological differences were minor. Hespenheide and Bellamy (2009) later describe these differences as dissimilarities in the size of spots of setae on the elytra as well as small differences on the pronotum. However, in response to the elevated levels of oak mortality in San Diego Co., California, Hespenheide and Bellamy (2009) re-evaluated the

taxonomic status of the invasive population in California and gave subspecies distinction to the two original species. These subspecies designations were *Agrilus coxalis coxalis* (representing the Mexico and Guatemala populations) and *Agrilus coxalis auroguttatus* (representing the Arizona and California populations). More recently, the taxa were examined again, and based on the morphology of the male genitalia Hespenheide et al. (2011) concluded that the Arizona/California and Mexican/Guatemalan forms do indeed comprise separate species (Coleman and Seybold 2011, Coleman et al. 2012a). Therefore, the populations in southern Mexico and Guatemala are recognized once again as *A. coxalis*, and the populations in Arizona and southern California are *A. auroguttatus*.

Distribution. The goldspotted oak borer (*A. auroguttatus*) (Fig. 1.1) is a buprestid beetle which is native to oak woodlands in southeastern Arizona, and perhaps northern Mexico (based on the single *A. auroguttatus* specimen collected in Baja California Sur). This invasive insect was first detected in San Diego Co., in 2004 by the California Department of Food and Agriculture (CDFA) during an exotic wood borer survey, but was not linked to oak mortality until 2008 (Coleman and Seybold 2008a). Goldspotted oak borer was probably introduced into southern California via the movement of infested oak firewood. Unregulated movement of firewood has accidentally moved numerous exotic wood borers into new areas of the United States (e.g., the emerald ash borer, *Agrilus planipennis* Fairmaire) (Haack et al. 2010). However, due to the proximity of *A. auroguttatus'* native Arizona range to southern California, Westcott (2005) hypothesized that the species naturally expanded its distribution into California.

Fig. 1.1. Goldspotted oak borer, *Agrilus auroguttatus*, adult collected in San Diego County (photo by Mike Lewis).

Natural range expansion is an unlikely explanation for the presence of *A. auroguttatus* in southern California. Geographical barriers such as the Mojave and Sonoran deserts make range expansion improbable because these regions lack suitable oak hosts required for *A. auroguttatus* development. From its native range in Arizona, *A. auroguttatus* would have to fly unassisted for approximately 600 km to reach suitable oak hosts in California. The separation of oaks by these two deserts has resulted in the geographical isolation of *A. auroguttatus* in Arizona. Additionally, the locations of initial infestation and the spread of oak mortality thereafter do not support range expansion (Fig. 1.2). The original populations of *A. auroguttatus* in southern California arose inland inside mature oak forests. Because the edges of infested oak woodlands were not attacked first, this observation is contrary to what would be expected from a natural range expansion from *A. auroguttatus* populations in Arizona or Mexico. The proximity of the initial California population to recreation areas such as the Laguna Mountain Recreation

Area in the Cleveland National Forest, Rancho Cuyamaca State Park, and William Heise County Park (all in San Diego County) further support the hypothesis that *A. auroguttatus* was introduced into southern California through firewood associated with outdoor recreational activities (e.g., camping). Additional support for this hypothesis are the two satellite infestations that were found in 2009 at Marion Bear Memorial Park in urban San Diego, (approximately 40 miles west of major infestation zones), and from Idyllwild, Riverside County, CA in 2012, which is approximately 80 miles north from the infestation epicenter (Jones et al. 2013). This most recent find in Idyllwild resulted in immediate eradication efforts by local government agencies including CAL Fire and the US Forest Service. It remains unclear whether eradication of *A. auroguttatus* in Idyllwild was successful.

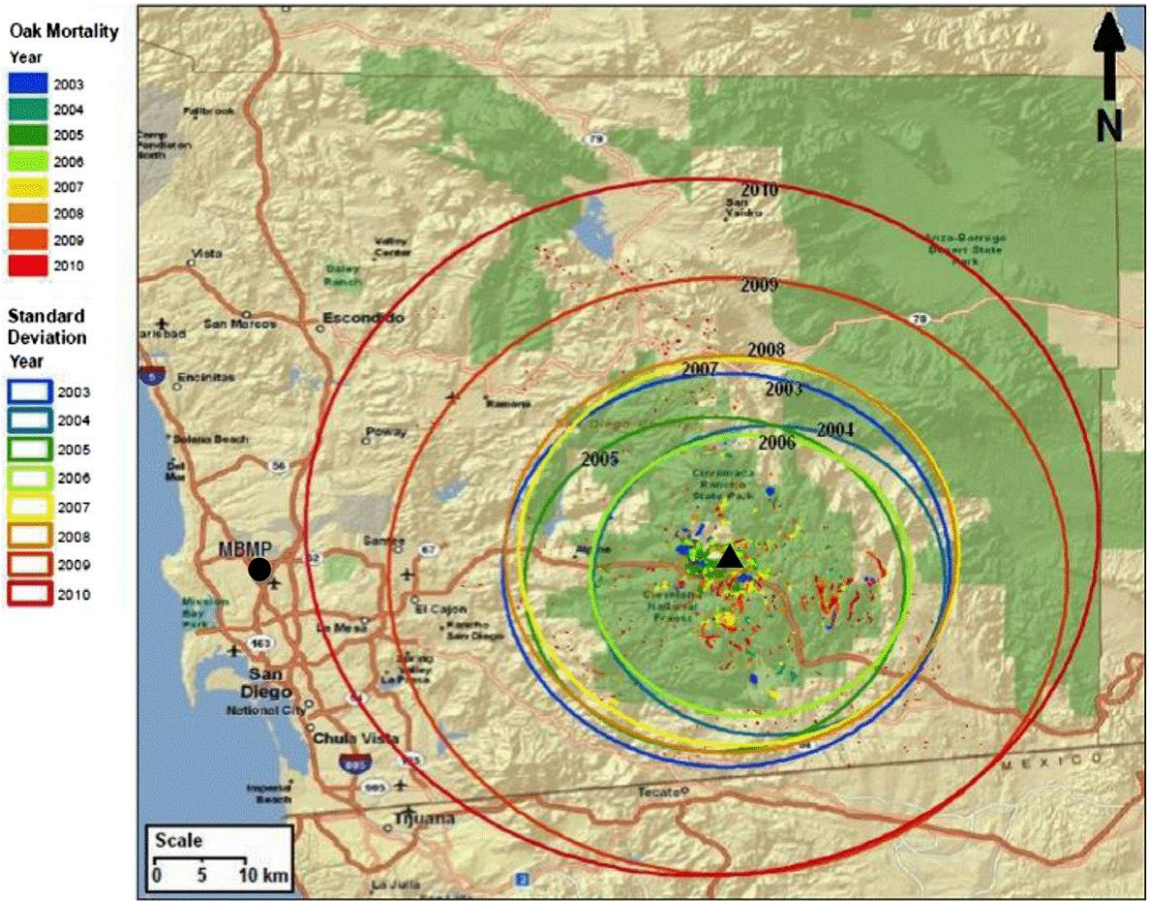

Fig. 1.2. Standard distance analysis of aerial oak mortality polygons (2003–2010) associated with *Agrilus auroguttatus* in San Diego Co., California, USA. The predicted center of oak mortality polygons from 2003–2010 is noted in the middle of the geometric means (▲). The known satellite infestations in Marian Bear Memorial Park (MBMP) north of urban San Diego is also presented (●). (Coleman et al. 2012b).

Currently, the known distribution of *A. auroguttatus* is throughout southern mountain ranges in Arizona (Cochise, Pima, and Santa Cruz Counties) as well as in San Diego and Riverside Counties, California (Fig 1.3). Historical and more recent collections indicate that *A. auroguttatus* occurs across the Chiricahua, Huachuca, Santa Catalina, Santa Rita (Coleman and Seybold 2011), and Dragoon mountains on the Coronado National Forest in Arizona. *Agrilus auroguttatus*' distribution in San Diego

Co. is estimated to cover 212,460 ha in the Cleveland National Forest, Descanso Ranger District. The infestation crosses all land ownerships in San Diego Co., including public, private, tribal, state, and county properties.

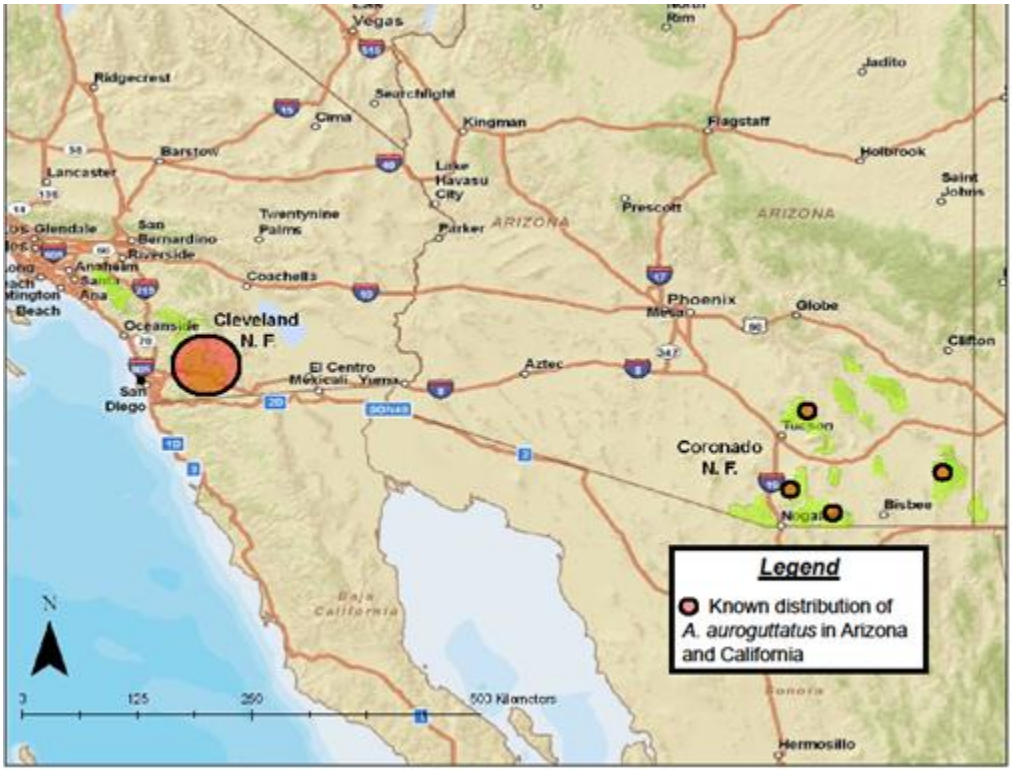

Fig. 1.3. Map of the known distribution of A. auroguttatus in Arizona and California (map by Tom W. Coleman).

Goldspotted Oak Borer Biology and Ecology

Biology. Before 2008, no information was available on the biology or life history of *A. auroguttatus* or *A. coxalis*. However, more recent studies in southern California suggest that most *A. auroguttatus* larvae complete their development to adulthood in a single year (Haavik et al. 2013). However, some larvae and pupae are present under bark in October, which indicates that individuals may require more than one year to complete development (Coleman and Seybold 2008a, Haavik et al. 2013). Adults emerge from May to October with peak flight activity from July through August (Haavik et al. 2013). *Agrilus auroguttatus* adults feed on oak foliage (Fig. 1.4) in the laboratory. However, leaf feeding is minor and has never been observed in the field. Mating has been observed on foliage in the laboratory, and it is likely that this behavior also occurs in the field. In the laboratory, females lay eggs individually on oak bark or artificial substrates, but *A. auroguttatus* eggs (Fig. 1.5) have rarely been observed in the field due to their small size (approx. 1 mm in width) and cryptic coloration. Based on information from other *Agrilus* species, it is likely that individual eggs are placed in bark crevices, which coupled with small size further compounds the difficult of visual detection in the field. Once eggs hatch, larvae burrow through the bark to reach the vascular cambium where they create feeding galleries in the outer phloem. Early and late instar larvae feed primarily in the cambial region (Fig. 1.6). When larval development is complete, larvae tunnel to the outer phloem to create pupal chambers underneath the outer bark. Larvae adopt a characteristic hair-pin configuration for pupation (Fig. 1.7).

Fig. 1.4. *Agrilus auroguttatus* adults feeding on oak foliage.

Fig. 1.5. *Agrilus auroguttatus* egg shortly after oviposition (placed onto an oak leaf for contrast).

Fig. 1.6. Late instar larva of *Agrilus auroguttatus* in its feeding tunnel.

Fig. 1.7. *Agrilus auroguttatus* larvae extracted from pupal chambers that are orientated into a hair-pin configuration.

Damage. *Agrilus auroguttatus* larvae create extensive feeding galleries where the phloem and xylem interfaces with the bark (Fig. 1.8). Feeding damage to the xylem and phloem prevents nutrient flow while simultaneously destroying the vascular cambium layer, the zone underneath the bark that gives rise to new xylem and phloem. The result is retardation of tree growth, and associated with *A. auroguttatus* infestation in oaks. These symptoms include crown thinning and die back, bark staining, D-shaped exit holes produced by emerging adult beetles, and woodpecker damage caused by birds foraging

for larvae (Hishinuma et al. 2011). Crown thinning and die back are typically the most readily observed symptoms, which begin with premature leaf drop and progress to twig and branch die back (Coleman and Seybold 2008a). Bark staining (Fig. 1.9) is the result of extensive larval feeding beneath the bark, which causes necrosis of subcortical tissues and can lead secondarily to infection by fungi. Bark staining appears as black and red stains or oozing sap, which may seep from under the bark or appear as blistering (Coleman and Seybold 2008b). Bark staining is a common symptom of *Agrilus* injury in hardwoods (Solomon 1995, Vansteenkiste et al. 2005).

Fig. 1.8. Feeding galleries of *Agrilus auroguttatus* larvae underneath the bark (photo by Tom W. Coleman).

Fig. 1.9. Staining on the bark surface of coast live oak, *Quercus agrifola*, resulting from *Agrilus auroguttatus* larval feeding (photo by Tom W. Coleman).

Typical of the genus, *A. auroguttatus* adults chew D-shaped exit holes through the bark after emerging from pupal cells located just beneath the bark surface. These exit holes (Fig. 1.10) are approximately 3 mm wide and indicate that larval activity has occurred within that particular region of the tree. Exit holes and bark staining are most common on the lower portion of the main stem (< 3 m). In California, the acorn woodpecker, *Melanerpes formicivorus* (Swainson) and Nuttall's woodpecker, *Picoides nuttallii* (Gambel) (both Piciformes: Picidae) forage for late instar larvae or pupae during the dormant months (i.e., October through May), and removal by these birds reveals larval galleries and pupal cells in the outer phloem (Fig. 1.11) (Coleman et al 2011). The presence of any life stage (larval, pupal, or adult) along with D-shaped exit holes or extensive wood pecker foraging on the main stem of oaks indicates infestation from *A. auroguttatus* in California (Hishinuma et al. 2011). Similar injury symptoms associated

with *A. auroguttatus* infestations are observed in oaks attacked by *A. coxalis* in southern Mexico (Coleman et al. 2012a).

Fig. 1.10. *Agrilus auroguttatus* adult exit holes.

Fig. 1.11. Woodpecker foraging damage due to *Agrilus auroguttatus* infestation.

Since its introduction into southern California, *A. auroguttatus* has caused widespread tree injury and mortality to three species of native California oaks; coast live oak (*Quercus agrifolia* Née), canyon live oak (*Quercus chrysolepis* Liebm.), and

California black oak (*Quercus kelloggii* Newb.). *Agrilus auroguttatus* has also been observed to colonize Engelmann oak (*Quercus engelmanni* Greene) at a very low frequency. However, mortality of this species caused from colonization by *A. auroguttatus* has not been observed, and infestation of *Q. engelmannii* is likely due to spill over from high populations of *A. auroguttatus* attacking more favored oaks species nearby. Tree mortality from *A. auroguttatus* has been estimated at >21,500 trees (Coleman et al. 2012a) (Fig. 1.12).

Fig. 1.12. Tree mortality in San Diego Co., California from *Agrilus auroguttatus* infestation.

Although *A. auroguttatus* was initially collected in southern California in 2004, oak mortality had been aerially mapped by the USDA Forest Service since 2002, which indicates that this beetle was probably introduced into the area several years before the

earliest documentation of oak mortality. Red oaks (section *Lobatae*) such as *Q. agrifolia* and *Q. kelloggii* appear to be preferred hosts for *A. auroguttatus*; while intermediate oaks (section *Protobalanus*) such as *Q. chrysolepis* are less preferred. White oaks (section *Quercus*) like *Q. engelmanni* are rarely attacked by *A. auroguttatus* in southern California, and injury to white oaks in Arizona from *A. auroguttatus* has never been documented (Coleman et al. 2012b). Coleman and Seybold (2011) hypothesized that phloem thickness, bark structure, and/or host chemistry within these different oak groups may influence host susceptibility to *A. auroguttatus*. Tree maturity also appears to affect the vulnerability of oaks to attack by these beetles since infestation and mortality is predominately observed in larger diameter trees (> 12 cm at breast height) (Coleman et al. 2012b).

The low levels of oak mortality in southeastern Arizona attributed to *A. auroguttatus* when compared to the elevated levels of mortality seen in San Diego Co. in California could be attributed to at least two factors, which together support the hypothesis that this wood borer is invasive in California. These factors are decreased host resistance and lack of co-evolved natural enemies in the invaded range of *A. auroguttatus* (Coleman and Seybold 2011). The varying levels of resistance by oak species native to Arizona and California may reflect evolutionary divergence and geographic isolation between these species and *A. auroguttatus*. Oak species in Arizona have experienced a much longer co-evolutionary period with *A. auroguttatus*, and may therefore be less susceptible to attack. California's oaks, on the other hand, have not co-

evolved with a species that causes similar damage, and therefore could be more vulnerable to injury from *A. auroguttatus* due to a lack of suitable defense mechanisms.

The oak woodlands of southern California are largely composed of species from the red oak group, which are highly vulnerable to *A. auroguttatus* infestation. The oaks attacked by *A. auroguttatus* are keystone species that hold dominant and co-dominant positions in the canopy. Therefore, their widespread loss is anticipated to result in detrimental effects on forest ecosystems in southern California. Biodiversity will likely be affected due to the loss of habitat and food resources for many native animals such as acorn woodpeckers (*Melanerpes formicivorus*), ground squirrels, mule deer (*Odocoileus hemionus*), specialist oak-exploiting insects, and the endangered arroyo toad (*Bufo californicus*). Oaks also supply shade to riparian areas and understory flora, which are critical components of these ecosystems. Also, the accumulation of dead oak litter from trees killed by borers will alter the fuel load in affected woodlands, increasing the probability and extent of wildfires (Coleman and Seybold 2008b).

The loss of oaks also affects local residents. Oaks are culturally significant to the indigenous people in this region who have traditionally utilized their acorns for food. Private landowners incur significant declines in property values when their oaks die. To maintain property values, homeowners will have to replace dead oaks or protect healthy uninfested trees. Arboricultural consultants estimate the value of a 20-yr-old coast live oak tree to be approximately $6,000, which equates to about $300 for each year the tree is

alive. Since the oak trees that are being killed by *A. auroguttatus* are typically very old (some are more than 100 years old), the value added to residential properties by a one hundred year old tree is conservatively estimated by property appraisers and professional arborists at > $10,000. Given such economic values for individual trees, the size of losses for whole towns, such as Descanso, Guatay, Julian, Pine Valley, and Ramona that are dominated by *A. auroguttatus*-infested oak woodlands, will clearly run into the millions or tens of millions of dollars ($USD).

Managing the Invasive Goldspotted Oak Borer in Southern California

Current Management Strategies. Methods to kill adults or brood of goldspotted oak borer include systemic and topical insecticides, wood solarization, and grinding of infested wood. The effectiveness of insecticide use for controlling *A. auroguttatus* is currently being tested. However, the use of insecticides to protect trees in forest stands is neither cost effective nor environmentally sensible. Pesticide use is best suited for private land owners or for saving heritage trees. Solarization (i.e., wrapping infested wood in plastic and heating in the sun to kill larvae or pupae in wood, or to trap and kill adults as they emerge) or grinding infested wood to kill larvae and pupae can provide localized control within an infested area, perhaps slowing expansion of the infestation. However, the most effective way to reduce spread is to stop the movement of infested firewood out of affected areas. Unfortunately, there are no mechanisms in place in California to prevent firewood movement at this time.

The lack of natural population control and the intolerance of California's oaks to herbivory from *A. auroguttatus* likely explain the high levels of tree mortality associated with *A. auroguttatus* infestations (Coleman and Seybold 2008b). Although short-term management practices such as the treatment of selected high value oaks with insecticides have been implemented, a long-term strategy is imperative in order to permanently bring the populations of this pest under control. Therefore, the development of a classical biological control program with co-evolved host specific parasitoids can potentially be a permanent, widespread, low cost, environmentally safe, and pest-specific management strategy.

Biological Control. The implementation of an effective classical biological control program for any invasive species requires the knowledge of several critical components such as the pest's biology, area of origin, natural enemies (in the home and introduced range), and life history traits. Prior to its introduction and subsequent invasiveness in southern California, *A. auroguttatus* was rarely collected and had a lack of data in the economic entomology literature (Coleman and Seybold, 2011). However, the rapid decline of southern California's oak forests from *A. auroguttatus* infestation has lead to increased research on the natural history, impact, and management of this wood-borer including surveys for co-evolved natural enemies for use in a classical biological control program in California (Coleman and Seybold 2008a, b, 2011, Coleman et al. 2011, 2012a, b, Haavik et al., 2012, 2013, Jones et al. 2013).

In 2009, natural enemy surveys were conducted in southeastern Arizona where previous *A. auroguttatus* collections were made (e.g., Santa Rita, Huachuca, Chiricahua, and Santa Catalina Mountain ranges), and on the Descanso Ranger District of the Cleveland National Forest in San Diego Co., California (Coleman and Seybold 2011). Additional surveys were conducted in 2010 in the southern Mexican states of Chiapas and Oaxaca. Field surveys (i.e., bark removal and extraction of larvae from infested trees) and rearing from bark collected in Arizona have identified the eupelmid parasitoid *Calosota elongata* (Fig.1.13) as a potential biological control agent for *A. auroguttatus* (Coleman and Seybold 2011). This gregarious ectoparasitoid was an unknown species when initially found on *A. auroguttatus* larvae and has been subsequently described (Gibson 2010). In Arizona, *C. elongata* was collected from *Q. emoryi* in Arizona at a 15% parasitism level (Coleman et al. 2012a). In December 2010, *C. elongata* was also found near the Pine Creek Trailhead, Cleveland National Forest in San Diego Co., California with a much lower parasitism rate of less than 1% (Haavik et al. 2012).

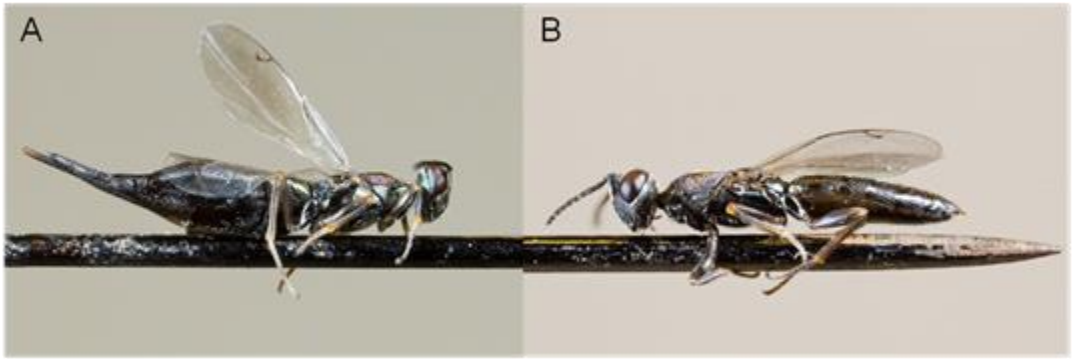

Fig. 1.13. *Calosota elongata* female (A) and male (B), a larval ectoparasitoid of *Agrilus auroguttatus*.

The life history for the potential biological control agent, *C. elongata*, is currently unknown, and molecular analyses would help to determine whether this parasitoid is native to southern California or was introduced into the area with *A. auroguttatus*. The effectiveness of *C. elongata* for the biological control of *A. auroguttatus* has not yet been assessed, but its distribution appears to be limited to areas occupied by *A. auroguttatus* in San Diego Co. It should be noted that this distribution may simply reflect intensive sampling for *A. auroguttatus*. *Calosota elongata* may naturally be widespread in California and has now reached detectible densities because of abundant hosts.

The remaining natural enemies reared from infested oak material or collected from *A. auroguttatus* life stages in Arizona were generalist predatory and parasitoid species that are not suitable for introduction into California as classical biological control agents. These include a bark gnawing beetle (Coleoptera: Trogossitidae), click beetle (Coleoptera: Elateridae), parasitic mite, *Pyemotes tritici* (Lagreze-Fossat and Montane) **(**Acari: Pyemotidae), *Atanycolus simplex* Cresson (Hymenoptera: Braconidae) (Coleman and Seybold 2011), and most recently in 2012, the first known egg parasitoid, a *Trichogramma* sp. (Lopez and Hoddle unpublished data).

In California, natural enemies of *A. auroguttatus* include a bark gnawing beetle (Coleoptera: Trogossitidae), *Pyemotes tritici*, *Atanycolus simplex*, and a snakefly larva (*Agulla* sp., Neuroptera: Raphidioptera) (Coleman and Seybold 2011). Bark gnawing beetles and *Agulla* sp. are generalist predators of bark beetles and wood borers that are

found throughout the western United States (Furniss and Carolin 1977). Additionally, *Atanycolus simplex* is a generalist larval parasitoid that has been associated with various buprestid and cerambycid beetles throughout the United States (Quicke and Sharkey 1989, Krischik and Davidson 2007, USDA Forest Service 2010).

Host specificity testing has not been conducted on any known natural enemies of *A. auroguttatus*. However, once potential biological control agents are identified and the respective rearing methods established, host range tests in quarantine will likely include native and non-native insects which are evolutionarily and behaviorally related to *A. auroguttatus*. Potential non-target test species may include the bronze birch borer (*Agrilus anxius* Gory), oak twig girdler (*Agrilus angelicus* Horn), twolined chestnut borer (*Agrilus bilineatus* Weber), emerald ashborer (*Agrilus planipennis*), and soapberry borer (*Agrilus prionurus*). Other non-congeneric species that occupy similar niches to *A. aurogutattus,* like nautical borer (*Xylotrechus nauticus* LeConte), and flatheaded appletree borer (C*hrysobothris femorat*a Olivier) may also be considered for testing. Together, these two test groups of non-target insects, *Agrilus* spp., and non-*Agrilus* spp., will test the specificity of *A. auroguttatus* natural enemies based on phylogenetic and ecological relatedness. There are no endangered or threatened species of Coleoptera in California that use oaks as hosts, and some of the species listed here can be pests (e.g., *C. femorata*). Species selections will be strongly determined by availability and ease of rearing.

Developing a Biological Control Program for Goldspotted Oak Borer

Recent studies on *A. auroguttatus* have helped to improve our understanding of this woodborer's natural history and impact in native and introduced areas (Coleman and Seybold 2008a, b, 2011, Coleman et al. 2011, 2012a, b, Haavik et al., 2012, 2013). However, further research on the general biology, life history, and potential natural enemies of *A. auroguttatus* is necessary in order to develop an effective biological control program for this invasive beetle. Consequently, in order to acquire the basic information needed to initiate a classical biological control program for *A. auroguttatus* in California, this dissertation research had the following four objectives: 1) determine the fecundity and longevity of *A. auroguttatus* under varying diet and mating treatments, 2) assess the dispersal capabilities of *A. auroguttatus* adults in the laboratory using computerized flight mills, 3) use molecular methods to identify the area of origin for the California population of *A. auroguttatus,* and 4) survey for natural enemies of this beetle by deploying *A. auroguttatus* egg masses into the native and introduced range.

Determining fecundity and longevity for goldspotted oak borer. Quantifying basic life history traits such as fecundity and longevity is essential for understanding the fundamental aspects of *A. auroguttatus* that can facilitate in the development of efficient, species-specific management strategies for this pest. The development of a classical biological control program for *A. auroguttatus* can be greatly enhanced by information on biology and life history. This knowledge can guide foreign exploration efforts, and aid in

the selection of natural enemies which will have the greatest deleterious impact on this beetle by targeting the most vulnerable life stages. Information about the occurrence and persistence of specific life stages, and the effects of adult size on the abundance and viability of offspring can also help with the development of mass rearing programs for the goldspotted oak borer which would enable the rearing of natural enemies for use in a classical biological control program against this pest. This information can also be used to assess factors influencing the phenology of natural enemies. Lastly, quantifying the potential fecundity and longevity of *A. auroguttatus* can benefit risk assessment models which rely on estimates of population growth and dispersal.

Assessing the Flight Capabilities of *A. auroguttatus* Using Computerized Flight Mills. Understanding the dispersal potential of *A. auroguttatus* in southern California is critical to the development of risk assessment and management plans, including biological control. However, assessing dispersal potential in woodborers is difficult due to the cryptic nature of these insects. Typically, dispersal of woodborers without known attractants (e.g., pheromones) is evaluated by destructively sampling trees around outlying infestation sites (Siegert et al 2010), which can be time consuming and laborious. Alternatively, knowledge of dispersal potential can be acquired through the use of computerized flight mills. Flight mills are a practical tool for understanding the flight capabilities of an insect within a controlled laboratory environment. Since the flight capabilities of *A. auroguttatus* were unknown, information gained from this research could be used in guiding releases of biocontrol agents within the infested areas

of southern California, and as groundwork for designing field dispersal studies which may provide a more realistic assessment of dispersal behavior in the wild. This information will also be a useful component in risk assessment models of which natural dispersal is an important component.

Determining the Area of Origin for Goldspotted Oak Borer. The area of origin of the California population of *A. auroguttatus* from within its large native range in southern Arizona was not known until studies reported here were conducted. DNA-based techniques were used to identify the area of origin of California's invasive population of *A. auroguttatus*. Identification of this area within the native range of GSOB can facilitate the collection of natural enemies that have evolved to exploit the genotype of the invading pest population. Additionally, determining the area of origin for the California population of *A. auroguttatus* may provide insight into its potential invasion routes which may be of use for developing management plans to mitigate unwanted future introductions of forest pests from within the Continental USA.

Surveying for Natural Enemies of A. auroguttatus in the Native and Introduced Range. The rapid increase in population densities of *A. auroguttatus* within the oak woodlands of southern California may in part be due to a lack of co-evolved natural enemies. A key step in the development of a classical biological control program for *A. auroguttatus* relies on the identification of host-specific natural enemies from the pest's area of origin. Cut oak branches with sentinel *A. auroguttatus* eggs masses were

deployed in southeastern Arizona and California to attract potential egg parasitoids of *A. auroguttatus*. Egg parasitoids have shown potential as biological control agents of *A. planipennis* (Liu et al. 2007), and these natural enemies, should they exist for *A. auroguttatus*, are of high interest for use in the emerging biological control program for this pest. Additional surveys in the introduced range of *A. auroguttatus* were also conducted to determine whether arthropod natural enemies are currently exploiting this resource in California.

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Chapter 2

Effects of Body Size, Diet, and Mating on the Fecundity and Longevity of the Invasive Goldspotted Oak Borer (Coleoptera: Buprestidae)

Abstract

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, (Coleoptera: Buprestidae), is an invasive wood-borer that was recently introduced into southern California from southern Arizona, and has caused the rapid mortality of thousands of native oaks. Biological control of *A. auroguttatus* is a management strategy of high interest, but is in its early stages, which is due, in part, to a lack of information on the basic biology and life history of *A. auroguttatus*. To address this shortcoming, studies were conducted in quarantine on the realized daily and lifetime fecundity of *A. auroguttatus* females which were determined by comparing oviposition and larval emergence rates of females subjected to different dietary and mating treatments. Longevity and body size was also recorded for male and female adults under these treatments. *Agrilus auroguttatus* oviposition and larval emergence was similar in females that were continuously paired with either one or two males, but virgin females laid significantly fewer eggs and had no larval emergence. The number of mates did not have an effect on *A. auroguttatus* adult longevity, but a carbohydrate-enriched diet increased both longevity and oviposition. There was no correlation between measurements of adult

body size and the majority of parameters measured for *A. auroguttatus* fecundity or longevity. Hind tibia length was correlated with total oviposition period.

Introduction

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, (Coleoptera: Buprestidae) is a serious pest of native oaks in southern California, U.S.A. Initially detected in San Diego County, California in 2004 (Westcott 2005), this invasive beetle was likely introduced into the area through the movement of infested oak firewood from its native range in southern Arizona. In Arizona, *A. auroguttatus* preferentially attacks oaks that are in decline, and has never been considered a significant forest pest (Coleman et al. 2012). The impact of *Agrilus auroguttatus* in southern California is much more severe than that observed in Arizona as this insect aggressively attacks and kills coast live oak (*Quercus agrifolia*), California black oak (*Q. kelloggii* Newb.), and canyon live oak (*Q. chrysolepis* Liebm) (Coleman and Seybold 2008a). Mortality of these native species is estimated at >22,000 trees within the 212, 460 ha infestation zone in San Diego (Coleman et al. 2011), and more recently (2012), Riverside County (Jones et al. 2013). Repeated feeding damage to the phloem/xylem interface by *A. auroguttatus* larvae eventually results in tree death (Coleman and Seybold 2008b).

Oak woodlands are unique environments in California that are comprised of diverse native flora and fauna, and they contain the highest levels of biological diversity of any broad habitat in the state (Allen-Diaz et al. 2007). As foundation species, oaks greatly influence the functioning of these ecosystems since they impact microclimates, affect energy and nutrient inputs, and provide essential habitat and food resources for a variety of native wildlife (Ellison et al. 2005). Oak habitat is also economically important in California as it enhances property values (Diamond et al. 1987), provides over two-thirds of range land for domestic livestock (Huntsinger et al. 1997), and it provides aesthetic and recreational value (Standiford and Tinnin 1996).

Management strategies for *A. auroguttatus* control are limited to protecting individual, highly valued trees (i.e., systemic and topical insecticides are used), preventing the spread of this insect through the sanitation of infested oak wood (i.e., wood solarization and grinding infested wood), or outreach efforts on the risks of moving infested firewood. At this time, biological control is the only feasible long-term method for controlling this pest on a forest scale because compared to pesticide use, biological control is less expensive, is not labor intensive, and is sustainable. However, developing a biological control program requires an understanding of the pest's fundamental biological and life history traits, of which nothing is known for *A. auroguttatus*. Prior to 2008, the only information available for *A. auroguttatus* was collection records from its native range in Arizona (Coleman and Seybold 2008b, Coleman and Seybold 2011).

The lack of information regarding the basic biology and life history of *A. auroguttatus* poses a significant challenge to our understanding, and ultimately management of this pest. Knowledge of the biology and life history of a pest can aid in the selection of natural enemies for biological control programs. Information about the occurrence and persistence of specific life stages, and the effects of adult size on the abundance and viability of offspring can help with the development of mass rearing programs and it may also guide foreign exploration programs for natural enemies which will have the greatest deleterious impact on the pest by targeting the most vulnerable life stages (Shea and Kelly 1998, Buhle et al. 2005). Additionally, quantification of its expected lifetime and reproductive capabilities will increase understanding of factors affecting *A. auroguttatus* population dynamics, which could be useful in risk assessment models that rely on estimates of population growth and dispersal (Venette et al. 2010).

Biological and life history information can also facilitate the development of mass rearing programs for *Agrilus auroguttatus*, or the mass production of a particular life stage. Currently, laboratory rearing of *A. auroguttatus* is restricted to the egg and neonate larval stages via field-collected adults or late-instar larvae that emerge as adults in the laboratory (Lopez, unpublished data). Methods for rearing *A. auroguttatus* to the adult stage have been investigated using emerald ash borer (*A. planipennis*) rearing techniques such as a modified emerald ash borer artificial diet (Blossey et al. 2000, Gould et al. 2011), as well as egg (L. S. Bauer, personal communication) and larval insertions (Duan et al. 2011, Duan and Oppel 2012) onto cut oak branches. However, none of these

methods were successful in rearing *A. auroguttatus* from the egg to adult stage. The ability to rear viable eggs in the laboratory has led to a focused search for egg parasitoids in Arizona as potential biological control agents of *A. auroguttatus*. Surveys for these parasitoids were initiated in 2012 using sentinel egg masses, and resulted in the collection of the first known egg parasitoid of this beetle, a currently undescribed *Trichogramma* species, a generalist collected in the native range of *A. auroguttatus* (Lopez and Hoddle in press). Determining the most effective methods for rearing *A. auroguttatus* eggs in the laboratory would help greatly the search for egg parasitoids by maximizing the number of sentinel eggs that can be deployed in both the native and introduced range of this beetle. Enhanced egg production techniques with field collected *A. auroguttatus* will also be advantageous for testing the specificity of potential biological control agents, and for mass rearing egg parasitoids for use in a classical biological control program against *A. auroguttatus* in California.

An insect's reproductive success can be affected by its size, nutrition, and access to mates. Body size has been shown to be correlated to fecundity in many insect species (Honěk 1993, French and Hammack 2010, Lauzière et al. 2000). Nutritional quality (i.e., diet) and mating frequency have also been shown to influence insect longevity and reproduction (Tisdale and Sappington 2001, Keena 2002, Paine et al. 2003, French and Hammack 2011). The objectives of the studies reported here were: (1) to determine the realized daily and lifetime fecundity of *A. auroguttatus* females by comparing oviposition and larval emergence rates of females subjected to different dietary and mating

treatments; and, (2) to investigate the relationship between longevity and body size in adult male and female *A. auroguttatus* maintained under different diet and mating treatments. This laboratory investigation under quarantine conditions provides the first detailed life history data for *A. auroguttatus*.

Materials and Methods

Rearing of *A. auroguttatus* Adults. *Agrilus auroguttatus* adults were reared from infested *Quercus agrifolia* and *Q. kelloggii* trees at two locations in southern California. In March-April 2011, infested trees were felled at various locations within the Cleveland National Forest, San Diego County, California (CA), cut into rounds (approximately 30 x 60 cm), and placed inside 15-30 emergence tents (1.83 x 1.83 x 1.83 m Lumite® screen portable field cages, Bioquip Products, Rancho Dominguez, CA) located at the Camp Ole U.S. Forest Service Fire Station, Mt. Laguna, CA. The following April (2012), infested *Q. agrifolia and Q. kelloggii* trees were felled at William Heise County Park, Julian, CA, USA (33°02'N/116°35'W; 1280 m), and similarly cut into rounds and tented at William Heise County Park in 2012. From June to August each year, adults were collected daily from the emergence tents. Immediately following collection, live adults were transported under permit (CDFA Permit No. = 2664; USDA-APHIS permit No. = P526P-10-00667) to the Insectary and Quarantine Facility at the University of California, Riverside County. In addition, in April 2012, bark was removed from *A. auroguttatus*-infested *Q. agrifolia and Q. kelloggii* trees at William Heise County

Park, and transported to the Insectary and Quarantine Facility at the University of California, Riverside under the same permits. Within the secure quarantine-rated laboratories, newly emerged adults were collected daily from the infested bark from May-June 2012.

Following field cage and quarantine collections, newly emerged *A. auroguttatus* adults (<24 hours old) were randomly selected for placement into treatment combinations that were assembled from a mixture of different mating and diet treatments. For every treatment combination, the emergence date of adults was recorded, and newly emerged adults were introduced into 2.13 L hand-grip rearing containers (11.7 x 12.1 x 18.1 cm, Candy Concepts Inc., Pewaukee, WI) held under ambient laboratory conditions (14:10 L:D, 23 ± 2 °C, 30 ± 2% RH) in quarantine, and where they remained until death. Each rearing container had a 6 cm diameter ventilation hole that was covered with fine metal mesh screen, and standard white coffee filter paper (11.1 cm diameter base, Ambiance™, Amerifoods Trading Co., Los Angeles, CA) was provided as an oviposition substrate. The coffee filter paper was cut into approximately 10 cm diameter rounds and placed directly underneath the rearing container lid. A fine metal mesh screen was placed on top of the filter paper which held the paper in place, secured the ventilation hole, and provided adults with a textured substrate for gripping while ovipositing. This method of acquiring eggs has been used successfully for research on emerald ash borer (*A. planipennis*) (Juli Gould, USDA-APHIS-PPQ, personal communication).

Experimental Design. A factorial design was used in this study. The two factors were diet type and number of mates, consisting of three and four levels, respectively. Covariates included three measurements of body size (elytron length, width, and tibia length). The four mating treatments were as follows: one male only (treatment 1 [T1]), one female only (treatment 2 [T2]), one female paired with one male (treatment 3 [T3]), and one female paired with two males (treatment 4 [T4]) to maximize sperm availability. For the duration of the study, adults within each mating treatment were randomly assigned to one of the following three diets: water only, water and *Q. kelloggii* foliage, and 10% honey-water solution with *Q. kelloggii* foliage. Condiment containers (59 ml, First Street™, Amerifoods Trading Co., Los Angeles, CA) with an approximately 1 cm diameter hole in the lid were used to hold each diet type. A cotton dental wick (3.8 x 1 cm, non-sterile Wrapped Rolls, Richmond Dental and Medical, Charlotte, NC) was placed into the lid hole which allowed adults to safely access diet liquids. *Quercus kelloggii* leaves used in specified diets were placed into condiment containers (holding respective diet liquids) through the lid hole along with a cotton wick. Diets (including condiment containers and dental wicks) were replenished every 3-4 days, a time interval shown in preliminary studies to be adequate.

Data Collection. The realized fecundity for each female was determined by removing the coffee filter paper daily (and replacing with fresh paper) and counting the number of eggs laid. Coffee filter papers were labeled by date and rearing container identification number, and were held in sealed Petri dishes (14:10 L:D, 23 ± 2 °C, 30 ±

2% RH) until larval emergence was complete, and larval emergence holes from eggs on each paper were counted. Egg counts were used to calculate average daily and lifetime oviposition for individual *A. auroguttatus* females in each of the four treatments. Since the emergence and oviposition period of females used in this study was sporadic, the number of eggs oviposited per day (total number of eggs oviposited/ total oviposition period) for each female was used to calculate the average daily oviposition for *A. auroguttatus* females in each treatment. Larval emergence (eclosion of eggs) counts were used to calculate lifetime and percentage of larval emergence for *A. auroguttatus* females in all treatments. Male and female longevity was also recorded daily for all treatments.

Body size of males and females from all treatments was estimated by removing the right hind tibia and right elytron of each adult, and slide mounting in a balsam/clove oil mix to measure elytron length and width, and tibia length. To slide mount each *A. auroguttatus* adult's tibia and elytron, a balsam mix was made from approximately 80% Canada balsam (CAS #8007-47-7) and 20% clove (bud) oil (CAS #80000-34-8). Two separate drops of the balsam mix (approximately 2 cm in diameter) were placed individually near the center of a plain glass microscope slide for placement of the tibia and elytron (each placed in the center of one drop). A cover slip was placed over the mounted specimens, and the labeled slide was placed into a slide warmer until the balsam mix solidified. After solidification, the elytron length, width, and hind tibia length were measured. Tibia length was measured using a stereoscope eyepiece micrometer

(calibrated with a stage micrometer), and elytron length and width were measured with an electronic 0-200 mm digital caliper.

Statistical analysis. Female oviposition (the number of eggs oviposited per day and per lifetime, time to initial oviposition, and total oviposition period), larval emergence, and adult longevity were analyzed using analysis of covariance (ANCOVA). The number of eggs laid per lifetime and adult longevity were analyzed on a natural logarithm scale, and time to initial oviposition and the number of eggs laid per day (eggs/day) were analyzed on an inverse scale to satisfy normality assumptions. Total oviposition period and larval emergence data was normally distributed. Females fed a water only diet had zero oviposition during this study and were therefore excluded from the oviposition data analyses. Additionally, larval emergence was analyzed using observations with non-zero oviposition data only. Tukey-Kramer was used to conduct pairwise comparisons to determine each significant factor in the ANCOVA. Finally, three measures of body size (elytron width, elytron length and tibia length) were independently analyzed to determine their potential correlation to fecundity and longevity. A t-test was used to compare the body size of male and female *A. auroguttatus* used in this study. All statistical analyses were conducted at the 0.05 level of significance and were performed using SAS 9.2 (SAS Institute Inc. 2008).

Results

There were no significant differences between 2011 and 2012 for the total number of eggs laid per lifetime ($F_{1,50} = 0.68$, $P = 0.42$), eggs/day ($F_{1,44} = 0.56$, $P = 0.46$), time to initial oviposition ($F_{1,47} = 0.94$, $P = 0.34$), total oviposition period ($F_{1,48} = 0.80$, $P = 0.38$), larval emergence ($F_{1,17} = 0.01$, $P = 0.94$) or adult longevity ($F_{1,161} = 0.29$, $P = 0.59$). The average (mean ± SE) lifetime and daily oviposition, lifetime oviposition period (days), days to first oviposition, lifetime and percentage larval emergence, and male (excluding treatment 1) and female longevity (days) are shown in Table 2.1. Male and female longevity was significantly different ($F_{1,161} = 7.35$, $P = 0.008$) with females living longer than males. The average longevity for treatment 1 males fed a water only ($n = 7$) diet was 12.4 ± 2.1 days. Males in treatment 1 fed a water and *Q. kelloggii* diet ($n = 8$) lived on average 53.9 ± 6.4 days, and males fed a 10% honey-water and *Q. kelloggii* ($n = 8$) diet lived 91.3 ± 17.2 days. Adult body size was measured from all males ($n = 87$) and females ($n = 80$) used in this study. The average body size for the population of males used was 7.03 ± 0.06 mm (elytron length), 1.20 ± 0.03 mm (elytron width), and 1.56 ± 0.01mm (tibia length). The average body size of females used was 7.73 ± 0.07 mm (elytron length), 1.31 ± 0.01mm (elytron width), and 1.68 ± 0.02 mm (tibia length). Females were significantly larger than males in elytron length ($t = 8.16$, df = 163, $P = \leq 0.0001$), elytron width ($t = 2.69$, df = 163, $P = 0.0079$), and tibia length ($t = 5.82$, df = 163, $P = \leq 0.0001$). The mean number of eggs oviposited by *A. auroguttatus* females paired with 0, 1, or 2 males, and fed water and *Q. kelloggii* foliage, or a 10% honey-water

and *Q. kelloggii* foliage diet is shown in Fig. 2.1. The mean number of larvae that emerged from eggs oviposited by *A. auroguttatus* females paired with 0, 1, or 2 males, and fed water and *Q. kelloggii* foliage, or a 10% honey-water and *Q. kelloggii* foliage diet is shown in Fig. 2.2. Larval emergence did not differ between mating or diet treatments. All females fed a water only diet laid zero eggs (Table 2.1), and were excluded from Fig. 2.1 and Fig. 2.2.

Table 2.1. Comparison of average (mean ± SE) fecundity and longevity characters for *A. auroguttatus* adults paired with either 0 (1♀), 1 (1♀x 1♂), or 2 (1♀x 2♂) males, and fed water only (H_20), water and *Q. kelloggii* foliage (H_20 & leaves), or a 10% honey-water and *Q. kelloggii* foliage (H-H_20 & leaves) diet.

Mean (± SE)	**1♀**			**1♀x 1♂**			**1♀ x 2♂**		
	H_20	H_20 & leaves	H-H_20 & leaves	H_20	H_20 & leaves	H-H_20 & leaves	H_20	H_20 & leaves	H-H_20 & leaves
Lifetime oviposition	0.0	38. 3 ± 18.6Aa	92.6 ± 32.3Ab	0.0	196.3 ± 49.1Ba	569.4 ± 99.6Bb	0.0	122.5 ± 30.9Ba	430. 7 ± 105.8Bb
Eggs/day	0.0	0.82 ± 0.3Aa	1.3 ± 0.3Ca	0.0	4.6 ± 0.9Aba	6.5 ± 0.3Da	0.0	2.0 ± 0.4Bab	6.5 ± 1.2Dac
Days to 1st oviposition	0.0	17.0 ± 5.1Aa	15.9 ± 3.7Aa	0.0	8.6 ± 0.9Ba	10.1 ± 0.7Ba	0.0	14.6 ± 2.8ABa	10.9 ± 2.3ABa
Total oviposition period (d)	0.0	43.0 ± 10.5Aa	63.5 ± 13.0Ab	0.0	43.6 ± 7.4Aa	85.8 ± 13.6Ab	0.0	53.8 ± 6.0Aa	77.7 ± 19.0Ab
Lifetime larval emergence	0.0	0.0	0.0	0.0Aa	171.8 ± 47.6Aa	393.5 ± 75.8Aa	0.0Aa	95.4 ± 26.1Aa	308.1 ± 92.6Aa
% Lifetime larval emergence	0.0	0.0	0.0	0.0	83.4 ± 4.0%Aa	72.6 ± 7.0%Aa	0.0	67.0 ± 10.2%Aa	69.2 ± 8.9%Aa
Female longevity (d)	17.8 ± 2.5Aa	83.8 ± 10.6Ab	106.8 ± 20.8Ac	16.5 ± 2.6Aa	72.1 ± 6.3Ab	122.1 ± 19.5Ac	13.3 ± 2.3Aa	75.6 ± 7.8Ab	109.1 ± 24.3Ac
Male longevity (d)	-	-	-	12.5 ± 2.1Aa	37.1 ± 6.0Ab	77.9 ± 20.1Ac	12.5 ± 1.7Aa	46.7 ± 5.3Ab	73.8 ± 13.8Ac
n	11	12	11	8	8	8	6	8	7

Means within each row followed by the same uppercase letter are not significantly different in mating treatment analyses, and means within each row followed by the same lowercase letter are not significantly different in diet treatment analyses based on a Tukey-Kramer means separation test with $\alpha = 0.05$ (SAS 2008).

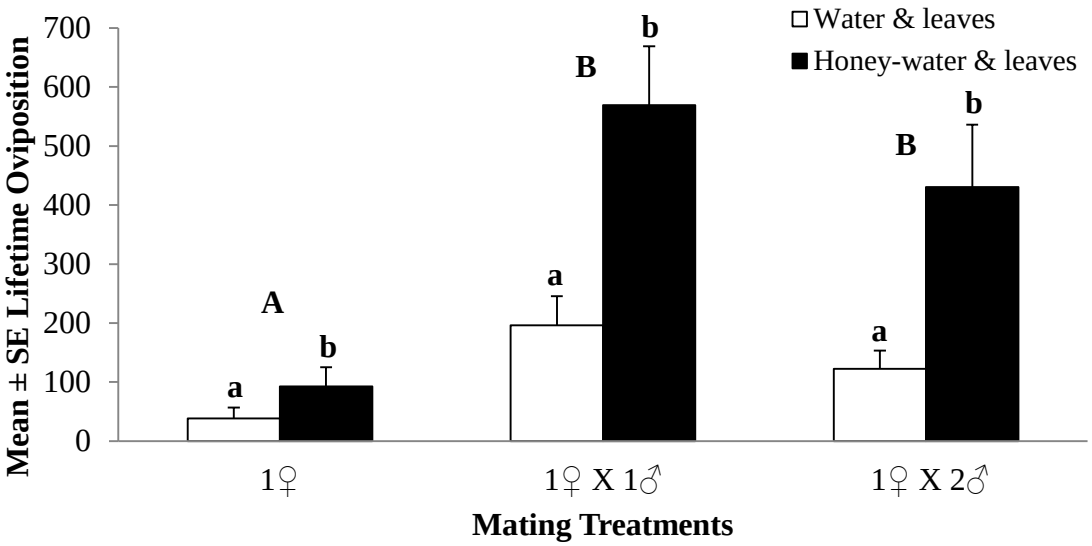

Fig. 2.1. Mean number of eggs oviposited by *A. auroguttatus* females paired with 0 (1♀), 1 (1♀ x 1♂), or 2 males (1♀ x 2♂), and fed water and *Q. kelloggii* foliage (water & leaves), or a 10% honey-water and *Q. kelloggii* foliage diet (Honey-water & leaves). Females fed a water only diet laid zero eggs throughout this study and were excluded from this figure. Different uppercase letters indicates a significant difference between mating treatments, and lowercase letters indicate a significant difference between diet types based on a Tukey-Kramer means separation test with $\alpha = 0.05$ (SAS 2008).

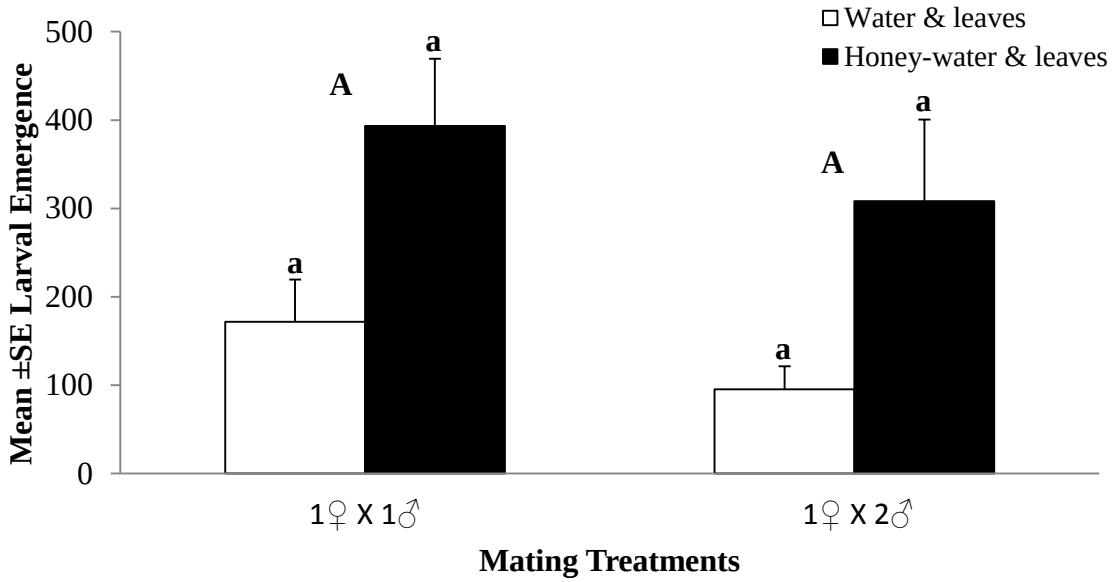

Fig. 2.2. Mean number of larvae that emerged from eggs oviposited by *A. auroguttatus* females paired with 0 (1♀), 1 (1♀ x 1♂), or 2 males (1♀ x 2♂), and fed water and *Q. kelloggii* foliage (water & leaves), or a 10% honey-water and *Q. kelloggii* foliage diet (Honey-water & leaves). Different uppercase letters indicate a significant difference between mating treatments, and lowercase letters indicate a significant difference between diet types based on a Tukey-Kramer means separation test with $\alpha = 0.05$ (SAS 2008).

Influence of the Number of Males on Female Fecundity and Adult Longevity.

The number of males paired with female *A. auroguttatus* had a significant effect on lifetime oviposition ($F_{2,50} = 16.38$, $P = \leq 0.0001$), eggs laid per day ($F_{2,44} = 32.99$, $P = \leq 0.0001$), and days to initial oviposition ($F_{2,47} = 9.20$, $P = \leq 0.0005$). Total oviposition period ($F_{2,48} = 0.07$, $P = 0.94$), and larval emergence ($F_{1,17} = 0.97$, $P = 0.34$) were not influenced by the number of males housed with each female. Pairwise comparisons using Tukey-Kramer showed that females with no access to males (T2) laid significantly fewer eggs over their lifetime than females with access to one (T3) ($t = -6.03$, df = 50, $P = \leq 0.0001$) or two males (T4) ($t = -4.42$, df = 50, $P = \leq 0.0001$). No larvae emerged from

eggs laid by unmated T2 females. The number of males paired with females had no significant effect on adult (male and female) longevity ($F_{3,161} = 0.41$, $P = 0.75$) across treatments. In general, adult longevity was significantly different between *A. auroguttatus* males and females ($F_{1,161} = 7.35$, $P = 0.008$), with females in all treatment combinations living longer than males across all treatments.

Influence of Diet on Female Fecundity and Adult Longevity. Diet type had a significant effect on lifetime oviposition (Fig. 2.1). Females fed 10% honey-water and *Q. kelloggii* leaves laid more eggs over their lifetime ($F_{1,50} = 15.49$, $P = 0.0003$) and had a longer oviposition period ($F_{1,48} = 5.74$, $P = 0.21$) than females fed a water and *Q. kelloggii* diet (Table 2.1). There was a significant interaction between mating and diet treatments for the number of eggs/day. Females fed water and *Q. kelloggii* leaves and paired with one male (T3) laid significantly more eggs/day than females paired with either zero (T2) ($t = -4.51$, df = 22, $P = 0.0005$), or two males (T4) ($t = 2.97$, df = 22, $P = 0.02$) (Table 2.1). However, when fed 10% honey-water and *Q. Kelloggii* leaves, single females laid fewer eggs/day than monogamous (T3) or polyandrous (T4) females ($t = -7.04$, df = 22, $P = \leq 0.0001$, and ($t = -6.46$, df = 22, $P = \leq 0.0001$, respectively). Diet type had no significant effect on larval emergence ($F_{1,17} = 1.51$, $P = 0.23$). Adult longevity was significantly affected by diet type ($F_{2,161} = 81.17$, $P = \leq 0.0001$). Male and female *A. auroguttatus* adults lived significantly longer when fed a 10% honey-water and *Q. kelloggii* leaves than a water and *Q. kelloggii* ($t = -5.10$, df = 161, $P = \leq 0.0001$), or water only diet ($t = -7.00$, df = 161, $P = \leq 0.0001$).

Influence of body size on Female Fecundity and Adult Longevity. Adult body size was not significantly correlated with the majority of measured fitness parameters. Lifetime oviposition, the main parameter used to assess fitness of individual *A. auroguttatus*, was not correlated with elytron length ($F_{1,50} = 0.05$, $P = 0.82$), width ($F_{1,50} = 0.34$, $P = 0.56$) or tibia length ($F_{1,50} = 0.03$, $P = 0.87$). The number of eggs/day and the time period until initial oviposition were not correlated with any *A. auroguttatus* body measurement ($P = \geq 0.05$). Tibia length however, was significantly correlated with total oviposition period ($F_{1,48} = 4.43$, $P = 0.04$). Additionally, elytron length, width, or tibia length did not have a significant relationship with larval emergence or adult longevity ($P = \geq 0.05$).

Discussion

Influence of Number of Mates on Female Fecundity and Adult Longevity. *Agrilus auroguttatus* females mate frequently when paired with males (Lopez, pers. obs.). Several hypotheses to explain the benefits of multiple matings by females include: (1) a need to replenish depleting sperm supplies (Walker 1980, Sakurai 1998), (2) to ensure viable sperm transfer (Ridley 1988), (3) to replace stored sperm that may be senescing or accumulating mutations (Tsubaki and Yamagishi 1991, Purdom et al. 1968), (4) to enhance sperm competition for increased fertilization (Walker 1980), or (5) to promote genetic variability among offspring (Caldwell and Rankin 1972, Williams 1975, Jennions and Petrie 2000,Fox 1993). A meta-analysis conducted by Arnqvist and Nilsson (2000)

determined that multiple matings by females increased realized lifetime fecundity by 30-70%, but analyses did not specifically determine the possible effects of a female mating multiple times with the same male (monogamy), or multiple times with different males (polyandry) (Bybee et al. 2005).

Although the study conducted by Bybee et al. (2005) determined that polyandry in female *Phoracantha semipunctata* (Coleoptera: Cerambycidae) was detrimental to reproductive success in comparison to monogamy, *A. auroguttatus* lifetime oviposition and larval emergence were similar in females that were continuously paired with either one or two males. In this laboratory study, the comparable fecundity of polyandrous and monogamous females suggests that the cost of mating with multiple males (i.e., male harassment and exceeding optimal mating frequency) may potentially cancel out any possible benefit gained from acquiring the additional sperm of a second male. Several benefits of polyandry in insects (e.g., genetic variability and vigor among offspring) can likely be quantified by examining the quality and reproductive fitness of offspring. However, due to the restraints of rearing this beetle from egg to adult in the laboratory, the reproductive success of *A. auroguttatus* monogamous and polyandrous-produced offspring was not investigated. Lastly, virgin *Agrilus auroguttatus* females laid significantly fewer eggs from which no larvae emerged. Even though virgin females were capable of oviposition, all eggs laid by these females were non-viable (non-viable eggs appear shrunken, shriveled, and lack melanization) and did not hatch.

The number of males did not have an effect on *A. auroguttatus* adult female longevity. Several studies have shown that the number of mates a female has access to may influence female longevity, either by increasing longevity through the benefits of male-derived nutrients (Fox 1993, Fox et al. 1995) or decreasing longevity due to repeated male sexual harassment (Alcock et al. 1978, Walker 1980, Rice 2000), or through toxic substances in male seminal fluids (Fowler and Partridge 1989, Chapman et al. 1995). The similar longevity of females paired with zero, one, or two males indicates that the potential benefits and/or costs of mating on *A. auroguttatus* female longevity are either absent or too low to be quantified.

Influence of Diet on Female Fecundity and Adult Longevity. Lifetime oviposition for *A. auroguttatus* was greatest when females were fed a diet of a 10% honey-water solution and *Q. kelloggii* foliage, was reduced to zero when water only was provided, and intermediate when females had access to water and *Q. kelloggii* leaves. Longevity was significantly greater when adults were fed a 10% honey-water and *Q. kelloggii* leaves. Sugar enriched diets are known to increase the fecundity (Benschoter and Leal 1976, Heimpel et al. 1997, Baggen and Gurr 1998, Marchioro and Foerster 2013) and longevity (Lauzière et al. 2000, Fadamiro and Baker 1999, Benschoter and Leal 1976, Haynes 1985, Tisdale and Sappington 2001, Simmons et al. 2012) of many insects, and is therefore a useful and cost effective addition to mass rearing procedures. *Agrilus auroguttatus* females had an approximate 245 - 350% increase in average lifetime egg production, and lived on average approximately 23 - 50 days longer (i.e., 46%

longer) when given the additional carbohydrate resource than females that were fed *Q. kelloggii* leaves and water only. The lack of oviposition from females given only water suggests that adult feeding on oak leaves is a necessary requirement for *A. auroguttatus* egg maturation and oviposition. On the other hand, the lack of diet-induced effects on *A. auroguttatus* larval emergence indicates that egg viability was not influenced by the consumption of additional carbohydrates in the 10% honey-water solution in combination with oak leaves.

Influence of Body Size on Female Fecundity and Adult Longevity. Lifetime oviposition, time to initial oviposition, total oviposition period, larval emergence, and male and female longevity were not influenced by *A. auroguttatus* adult body size (i.e., elytron length or width, or hind tibia length). However, tibia length was marginally correlated with the number of eggs oviposited per day. According to Honěk (1993), female size is usually a good indicator of potential fecundity, although there are studies where no significant relationships were found (Slansky 1980, Boggs 1986, Johnson 1990, Ohgushi 1996, Klingenberg and Spence 1997). Large body size can enhance reproductive success in adults through physiological and behavioral advantages such as increased egg load, egg size (Ellers 1998, O'Neill and Skinner 1990), and competition for mates (Hanks et. al 1996, Forslund 2000). Adult body size and fecundity in insects is genetically determined and modified by environmental conditions during development, which may influence reproduction and growth (Honěk 1993). Body size can also influence longevity, although factors such as temperature, diet, and the overall health of

an individual may play a greater role (Carroll and Quiring 1993, Nylin and Gotthard 1998, Sokolovska et. al 2000). Since *A. auroguttatus* adults were reared from a number of different oak hosts growing in the Cleveland National Forest, the environmental conditions during larval development (i.e., host quality) are unknown, but may have had a similar influence on fecundity and longevity recorded from smaller and larger *A. auroguttatus* adults.

Information on the basic aspects of the reproductive biology of *A. auroguttatus* is the foundation for developing species-specific management strategies targeting this pest, and for understanding and assessing factors affecting invasion risk into new areas. At this time, classical biological control is seen as the best option for managing *A. auroguttatus* in a forest environment since the spread of this pest has become too great to control with methods such as eradication or pesticide applications. Surveying for and identifying suitable egg parasitoids of *A. auroguttatus* from its home range in southern Arizona using sentinel egg masses is currently the major focus in the development of a biological control program for this pest. Our inability to rear *A. auroguttatus* from eggs to adults in quarantine has changed the focus of the emerging biological control program from larval and pupal parasitoids found during surveys in Arizona (Coleman and Seybold 2011, Coleman et al. 2011) to prospecting for egg parasitoids. Although, almost nothing is known about the parasitoid fauna associated with *A. auroguttatus* eggs, this life stage is easily obtainable in the laboratory from field collected adults and can be easily and rapidly deployed at appropriate field sites in Arizona as sentinel egg masses before being

returned to quarantine to rear out parasitoids. The results of work presented here demonstrate that to maximize the fecundity and longevity of *A. auroguttatus* females for mass rearing eggs in the laboratory pairing females with one male and providing adults with a continuous diet of 10% honey-water and oak foliage is recommended. Further investigation into *A. auroguttatus* biology, such as the fecundity of singly-mated females and the influence of different temperatures on adult longevity and egg production, will help to determine whether continuous mating is necessary and the optimal temperatures to maximize fecundity and subsequent egg hatch.

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Chapter 3

Assessing the flight capabilities of the goldspotted oak borer, *Agrilus auroguttatus* (Coleoptera: Buprestidae), with computerized flight mills

Abstract

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, is an invasive wood-boring beetle that has caused considerable mortality to native oak species in southern California. Assessing the dispersal capabilities of this woodborer can help to determine its potential environmental and economic risk within the invaded region, and possibly allow the development of species-specific management strategies. The flight performance of *A. auroguttatus* adults under different age, mating, and nutritional status was assessed by tethering individuals to computerized flight mills for a 24-hr trial period to collect information on total distance, flight time, velocity, number and duration of flight bouts, and post-flight weigh. The nutritional status and body size (elytron length) of *A. auroguttatus* adults had a significant influence on overall flight performance. Mating status and gender were not important to many parameters of flight performance investigated here including total flight distance, duration, velocity, and flight bout time. Significant interactions between nutrition and age were observed in the overall flight performance, resulting in decreased flight activity in old, starved individuals during the 24-hr trial period. These results support the hypothesis that human-aided transportation

likely caused the initial introduction of *A. auroguttatus* into southern California's oak woodlands.

Introduction

When introduced insects become pestiferous in adventive areas, studies examining their dispersal capabilities can aid in determining the biological and abiotic factors that may influence rates of spread and establishment into new areas. Invasive species, such as insect pests, can be particularly damaging to forest ecosystems, causing severe economic and environmental impacts (Pimentel et al. 2000; Holmes et al. 2009**;** Lovett et al. 2006; Kenis et al. 2009). Millions of dollars are spent annually managing these forest pests because their negative effects on invaded ecosystems are often widespread and long-lasting (Pimental et al. 2005; Kovacs et al. 2010; Van Driesche et al. 2010). Therefore, identifying and understanding the traits that promote the proliferation and expansion of invasive species in natural forest ecosystems can help to determine the potential environmental and economic risk these species impose, and possibly allow the development of species-specific management strategies that mitigate their negative effects.

The goldspotted oak borer (*Agrilus auroguttatus* Schaeffer) is a small (adults are approx. 10 mm in length) invasive wood boring pest of native oaks within urban and natural environments in southern California (CA), U.S.A. Indigenous to southern

Arizona (AZ), U.S.A., *A. auroguttatus* was likely introduced into southern California through the transportation of infested oak firewood, and was initially detected in the Cleveland National Forest, San Diego County, CA in 2004 (Westcott 2005). In this area, approximately 212,460 ha of native forest are infested with this pest and it has been estimated that > 22,000 coast live oak (*Quercus agrifolia* Née), California black oak (*Q. kelloggii* Newb.), and canyon live oak (*Q. chrysolepis* Liebm.) have been killed because of larval feeding damage to the phloem/xylem interface (Coleman et al. 2012a).

Due to the proximity (approx. 550 km) of *A. auroguttatus'* native Arizona range to southern California, Westcott (2005) hypothesized that this species naturally expanded its distribution into California. However, this seems unlikely due to significant geographical barriers such as the Mojave and Sonoran deserts which lack suitable oak hosts required for adult feeding (oak leaves) and larval development (live oak trees in the red oak group, section *Lobatae*, with trunk diameters > 12 cm).

In fall 2012, a satellite infestation was discovered in the town of Idyllwild, Riverside County, CA, approximately 70 km north of the closest known infestation (Jones et al. 2013), and was shortly thereafter confirmed to be *A. auroguttatus* using molecular techniques (Lopez and Hoddle, unpublished data). Another satellite infestation was found in 2009 at Marion Bear Memorial Park in urban San Diego, CA, approximately 32 km west of major infestation zones (Coleman et al. 2012b). It is unknown how *A. auroguttatus* spread to these new areas, but the movement of infested

wood is the most likely explanation for the appearance of these isolated populations. The proximity of initial California populations to recreation areas such as the Laguna Mountain Recreation Area in the Cleveland National Forest, Rancho Cuyamaca State Park, and William Heise County Park (all in San Diego County with recreational camping facilities) support the hypothesis that *A. auroguttatus* was introduced into southern California through firewood associated with outdoor recreational activities (e.g., camping). Although firewood movement appears to be a major dispersal mechanism for *A. auroguttatus*, information on the natural flight potential of this beetle is lacking.

Flight mills are a practical tool for gaining insight into the flight capabilities of an insect due to their easy use within a controlled environment. However, because the behavioral effects of handling during attachment to the flight mill are largely unknown, and difficult to define and quantify, flight mill results may need to be interpreted with caution (Taylor et al. 2010). An approach for overcoming this challenge may be to simply compare the performance of two or more experimental groups of comparable size (Rowley et al. 1968). Through comparison, insight into the effects of biotic conditions (i.e., sex, mating status, age, etc.) on flight performance is obtained and can help in the overall assessment of an insect's dispersal potential. Another criticism of flight mill studies is that they may not be truly representative of flight performance in the field since they are conducted in the laboratory, and thereby lack the environmental stimuli normally encountered by a flying insect in the wild (Taylor et al. 2010). Although laboratory studies lack environmental components that potentially influence flight behavior, they

can be useful for research attempting to quantify basic attributes such as the frequency and periodicity of flight bouts, velocity, and duration of flight. These types of data are almost impossible to reliably collect in the field. At a minimum, flight mill studies provide estimates (with measures of variation) of certain flight related characteristics that can later be used for designing field dispersal studies and interpreting data from these experiments.

Dispersal characteristics of invasive insects exhibiting variable traits such as age, sex, and mating status may help enhance risk assessment analyses that are attempting to develop effective management strategies, such as the implementation of quarantine zones around newly infested areas (Landis, 2004; Neubert and Parker, 2004; Lodge et al. 2006; Jongejans et al., 2008; Sarvary et al., 2008; Koch et al. 2009). Because the flight capabilities of *A. auroguttatus* were unknown, adults of varying age, mating, and nutritional status were flown on flight mills to determine their combined effects on flight performance. This flight mill study is the first to evaluate the dispersal or flight potential of *A. auroguttatus*.

Materials and methods

Collection and Rearing of *A. auroguttatus* Adults for Flight Mill Studies.

Field-collected *A. auroguttatus* adults were reared from infested *Q. agrifolia and Q. kelloggii* trees that were felled in April 2012 at William Heise County Park, Julian, CA,

cut into rounds (approximately 30 x 60 cm), and placed inside 15 emergence tents (located at William Heise County Park). During June to August 2012, adults were collected daily from emergence tents (1.83 x 1.83 x 1.83 m Lumite® screen portable field cages, Bioquip Products, Rancho Dominguez, CA) containing approximately 15 *A. auroguttatus*-infested oak rounds. From these daily collections, adults were immediately sexed (Coleman and Seybold 2010), and randomly assigned to one of 16 experimental treatments shown in Table 1. Following treatment designation, adults were placed in 1 liter hand-grip rearing containers (9.2 x 9.5 x 14.6 cm, Candy Concepts Inc., Pewaukee, WI) until flown. Rearing containers had a 4 cm diameter ventilation hole that was covered with fine metal mesh screen, and were held under ambient laboratory conditions (14:10 L:D, 24 ± 4°C, 35 ± 5 % RH).

Designation of *A. auroguttatus* Adults to Experimental Treatments. The treatment combinations shown in Table 3.1 were created by immediately designating field collected male and female *A. auroguttatus* adults into one of each of the following categories: starved or fed, virgin or mated, and young or old. Adults in starved treatments were placed into rearing containers without food or water until flown. Fed adults were placed inside rearing containers provided with host plant leaves (*Q. kelloggii*) which was replaced every 3-4 days, and a moist cotton wick as a water source. To obtain virgin beetles, adults which emerged inside their respective rearing tents without others of the opposite sex were placed individually into rearing containers upon collection. Mated individuals were acquired by placing 1 male and 1 female together inside a rearing

container until copulation was observed and documented. Typically, copulation occurred shortly after adults were paired, although occasionally (approx. 25% of matings), several days of observation were needed to confirm mating had occurred. Adult beetles that were less than 2 days old were considered 'young', and adults that were more than 5 days of age were considered 'old'.

Description of Computerized Flight Mill. Seven flight mills were set-up inside a mobile laboratory (14:10 L:D, 24 ± 4°C, 35 ± 5 % RH) located at the William Heise County Park in San Diego County, CA. Each flight mill was connected to a laptop computer via a USB4 Encoder Data Acquisition Device (US Digital, Vancouver, WA), which was operated with customized software that collected and recorded flight activity. Raw flight data were summarized using customized macros in Microsoft Excel which calculated average velocity, total flight duration, number of flight bouts (movement of more than 5 seconds before coming to a complete stop), and distances flown during each flight bout and the total 24 hr trial period by individual *A. auroguttatus* adults.

Recording of Flight Measurements. To measure and record insect flight, a quadrature encoder (model 7700, Gurley Precision Instruments, NY) inside each flight mill (Fig. 3.1) provided both timing and arc travel direction information from flight activity (described below), which was used to calculate parameters of flight including velocity, distance, and duration. The encoder (Fig. 3.1) is comprised of a 33 mm diameter transparent disc with an opaque pattern of slits extending 2 mm radially near the edge

(Fig. 3.2). The slit pattern allows an IR source to pass through the disc to optical sensors. Two sensors (*A* and *B*) (Fig. 3.2) are positioned in such a way that if the disc travels clockwise, the rising edge of the *A* pulse is measured first, followed by the rising edge of the *B* pulse. When the disc is travelling counter-clockwise the B pulse is detected first. A third (*Z*) detector sees a single index slit placed on an inner concentric track to mark the 0 position of the disc, which gives a reference, or home position.

As the disc rotates, the slits pass over the sensors and a square-wave pulse train is generated. The resulting signal pulse train is read by a USB4 Encoder Data Acquisition Device (US Digital, Vancouver, WA). The acquisition device counts the pulse number since the last index was sensed, and clocks the time delta between rising edges with an internal frequency generator. Flight data was generated by attaching the insect to the encoder shaft (Fig. 3.1) by means of a 0.5 mm carbon steel flight mill arm (FMA) wire (30.5 cm in length), described below. The FMA was supported by the encoder shaft with a coupling (Fig. 3.1), and an equal length of the FMA wire passed through the coupling.

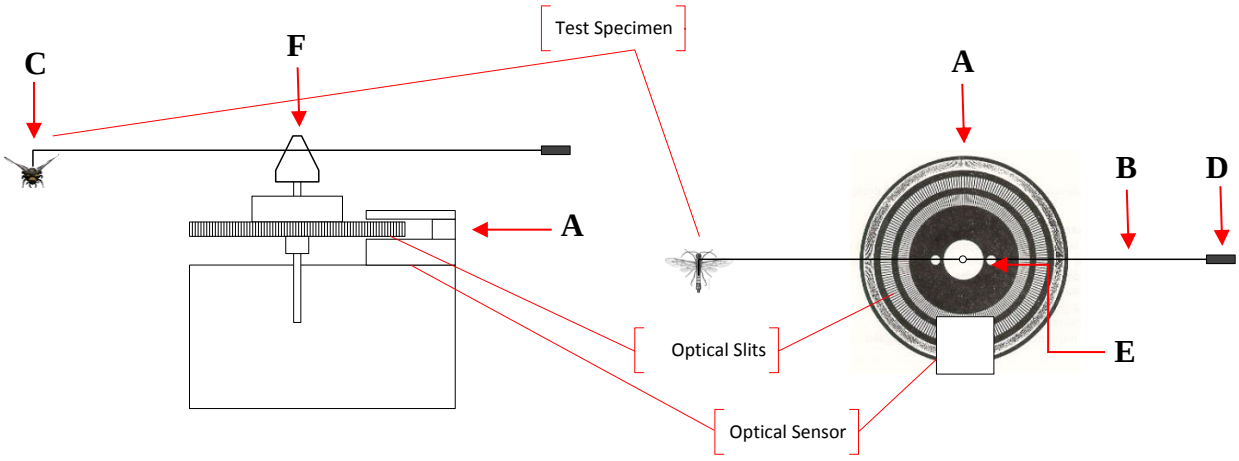

Fig. 3.1. Diagram of flight mill used to investigate the flight capabilities of *Agrilus auroguttatus*. The encoder (including the encoder disc's optical slits and sensor) (A), flight mill arm (FMA) (B), L-shaped flight mill arm attachment (C), counterbalance (D), encoder shaft (E), and encoder coupling (F) are illustrated.

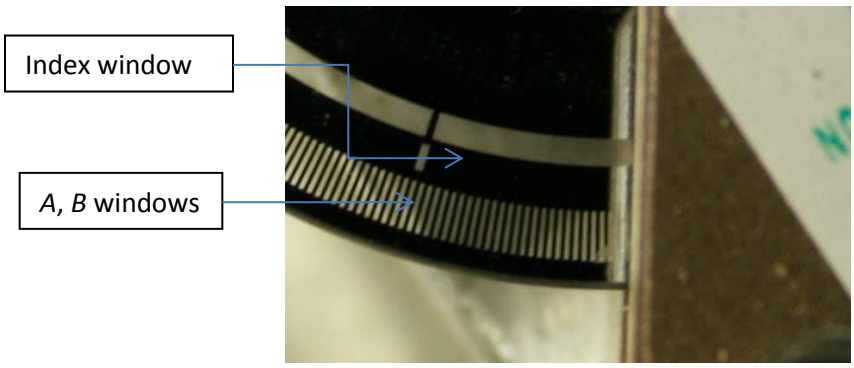

Fig. 3.2. Encoder disc showing outer ring and inner index track.

For every complete revolution, the end of the flight mill arm travels a distance approximately equal to the circumference of a circle whose radius equals the length of the FMA from the encoder shaft (center of the circle) to the specimen. This distance is calculated by the equation

$$C = 2\pi r \qquad \text{n.n}$$

Where

C circumference of the circle,

π the constant 3.1415926535…

r radius of the circle.

The encoder returns values between 0 and 500 depending on the position of the arm. Since the pulse value 0 (at the encoder index) is equal to 0 radians and the pulse value 500 is equal to 2π radians, these values are converted to radians using the equation

$$\theta = \frac{2\pi \cdot x}{500} \qquad \text{n.n}$$

Where

θ angle in radians

x pulse value.

To determine the distance traveled between each pulse, the difference between the latest pulse value and the previous pulse value is found. Direction of the flight mill arm's movement (i.e., clockwise or counterclockwise) is taken into account when calculating

this difference (note that individual insects are only able to fly in one direction once they are connected to the flight mill arm). The resulting value is converted into radians and the arc length for the angle is calculated using the equation

$$L = \theta r \qquad \text{n.n}$$

Where

L arc length

θ angle in radians

r radius of the circle.

The rate at which the end of the flight mill arm travels for the time period between the latest pulse and the previous pulse is calculated from this distance value and the time difference between the latest pulse and the previous pulse.

Calibration of Flight Mills. In order to estimate the accuracy of the pulse counter, its output was compared to a second calibration pulse counter with 5 times the resolution. The test unit pulse counter and the calibration pulse counter were linked and driven at the same speed by a motor. Pulse frequencies for both pulse counters were measured over a period of time with a TLS 216 Logic Scope (Tektronix, Beaverton, OR).

The TLS 216 was calibrated with an HP pulse generator (model 3324A, Hewlett Packard, Boeblingen, Germany).

For each sample, the ratio of the calibration frequency to the test unit frequency was calculated and compared to the expected ratio of 5.00. Ninety samples were obtained from 3 randomly selected test units at 3 motor speeds. The mean ratio was 5.0039, with a relative standard deviation of 1.97% and a relative standard error of 0.21%, confirming the accuracy of pulse information generated by flight mills.

Attachment of *A. auroguttatus* Adults to Flight Mills. Following treatment designation, adults were weighed with a precision balance (model GF-600, A&D Company Ltd., Milpitas, CA), then tethered by the pronotum to the arm of a flight mill (Fig. 3.3) via a FMA attachment (Fig. 3.2) and left for 24 hours. A piece of modeling clay was weighed and placed at the opposite end of the FMA to counterbalance the weight of each tethered adult (Fig. 3.2). To attach the insect to the FMA, *A. auroguttatus* adults were attached by the pronotum, using high strength glue (Gorilla Glue Company, Cincinnati, OH), to a small (0.59 mm diameter x 28 mm long) L-shaped wire that was flattened at one end. The L-shaped wire with the attached beetle was connected to a standard socket crimp (model 809-043, Glenair, Glendale, CA), which was directly connected to the FMA. After gluing and prior to flight data being recorded, free wing movement was visually confirmed. After a 24 hr flight trial, adults were detached from flight mills, immediately weighed, and elytron length was measured with an electronic 0-

200 mm digital caliper. After the 24 hour flight trial, any dead *A. auroguttatus* adults were discarded and excluded from the analyses, though this was rare (> 5 % of beetles flown).

Fig. 3.3. Adult GSOB tethered by the pronotum to a flight mill.

Statistical analysis. The flight performance of *A. auroguttatus* adults under varying age, mating, and nutritional status was analyzed using an analysis of covariance (ANCOVA). Covariates included a measurement of adult body size (elytron length) and weight. Measurements of flight performance included: total distance flown (m), total flight time (min), average flight velocity (m/s), number of flight bouts, and average flight bout time (s). For analyses of research variables, model assumptions were satisfied by conducting the following mathematical transformations (where y = research variable): total distance flown: $y^{0.3}$; total flight time: $y^{0.35}$; average velocity: $y^{0.4}$; number of flight bouts: $y^{0.5}$; and average flight bout time: $y^{-0.5}$. When significant interactions occurred, contrasts to determine the effects of interacting variables were conducted at the 0.05 level of significance. All statistical analyses were conducted at the 0.05 level of significance and were performed using SAS 9.2 (SAS Institute Inc. 2008).

Results

Total flight distance. The total distance flown by *A. auroguttatus* adults during each 24 hour flight period was significantly influenced by nutritional status ($F_{1, 177}$ = 62.53, $P = \leq 0.0001$) and body size ($F_{1, 177}$ = 48.82, $P = \leq 0.0001$), with larger, fed adults flying greater distances than smaller, starved individuals (Table 3.1 and Table 3.2). Pre-flight weight, mating status, and gender did not have an effect on the total distance flown during the trial period ($P = \geq 0.05$). A significant interaction between nutrition and age ($F_{1, 177}$ = 37.74, $P = \leq 0.0001$) existed; old fed adults flew further than old starved adults ($F_{1, 177}$ = 103.83, $P = \leq 0.0001$). Nutritional effects were not significant in the total flight distance of young *A. auroguttatus* adults ($F_{1, 177}$ = 1.61, P = 0.21).

Total flight duration. The total flight time of *A. auroguttatus* adults during each 24 hour trial period was significantly affected by nutritional status ($F_{1, 177}$ = 17.88, $P = \leq 0.0001$) and body size ($F_{1, 177}$ = 20.77, $P = \leq 0.0001$), with larger fed adults flying longer than smaller starved individuals. Pre-flight weight, mating status, and gender had no significant effect on total flight duration ($P = \geq 0.05$). There was a significant interaction between nutrition and age ($F_{1, 177}$ = 14.9, P = 0.0002). Age had an effect on total flight duration when adults were starved ($F_{1, 177}$ = 12.88, P = 0.0004), but not when adults were fed ($F_{1, 177}$ = 3.52, P = 0.06). However, the effects of nutrition (for both starved and fed adults) on total flight time was significant only in old individuals ($F_{1, 177}$ = 34.47, $P = \leq 0.0001$), which flew less during the 24 hour trial period when starved.

Average velocity. The average velocity of *A. auroguttatus* adults during flight was significantly affected by nutritional status ($F_{1, 177} = 37.90$, $P = \leq 0.0001$) and body size ($F_{1, 177} = 23.75$, $P = \leq 0.0001$), with larger, fed adults flying faster. There was no significant effect on total flight duration due to pre-flight weight, mating status, or gender ($P = \geq 0.05$). However, there was a significant interaction between nutritional status and age ($F_{1, 177} = 14.02$, $P = 0.0002$). Age had an effect on average velocity when adults were starved ($F_{1, 177} = 13.65$, $P = 0.0003$), but not when adults were fed ($F_{1, 177} = 2.53$, $P = 0.11$). However, the effects of nutrition (for both starved and fed adults) on average flight velocity was significant only for old individuals ($F_{1, 177} = 51.62$, $P = \leq 0.0001$) which had lower average flight velocities when starved. The effects of nutrition were not significant in young *A. auroguttatus* adults ($F_{1, 177} = 2.90$, $P = 0.09$).

Total number of flight bouts. The total number of flight bouts in each 24 hr flight trial was significantly affected by nutritional status ($F_{1, 173} = 6.15$, $P = 0.01$) and body size ($F_{1, 173} = 10.81$, $P = 0.0012$). Pre-flight weight had no significant effect on total flight duration ($P = > 0.05$). Significant interactions were observed between nutritional status and gender ($F_{1, 173} = 6.13$, $P = 0.01$), age ($F_{1, 173} = 9.48$, $P = 0.002$), and mating status ($F_{1, 173} = 5.16$, $P = 0.02$). The effects of gender on the number of flight bouts were only observed in fed adults ($F_{1, 173} = 4.66$, $P = 0.03$). Nutrition effects were significant for male *A. auroguttatus* ($F_{1, 173} = 11.84$, $P = 0.0007$), but not for females. Alternatively, the significant effects between nutrition and age were observed in starved ($F_{1, 173} = 8.36$, $P = 0.004$), old ($F_{1, 173} = 16.18$, $P = \leq 0.0001$) individuals irrespective of

gender. Finally, the interaction between nutritional and mating status showed significant effects in mated individuals ($F_{1, 173} = 11.89$, $P = 0.0007$), which also had a lower number of flight bouts than virgin adults when deprived of food.

Average flight bout time. The average time individual *A. auroguttatus* adults flew during each flight bout was significantly influenced by nutritional status ($F_{1, 177} = 31.10$, $P = \leq 0.0001$) and body size ($F_{1, 177} = 12.84$, $P = 0.0004$). However, there was no significant effect on total flight duration due to pre-flight weight, mating status, or gender ($P = \geq 0.05$). Significant interactions were observed between nutritional status and age ($F_{1, 177} = 14.41$, $P = 0.0002$). The effects of age were significant in starved ($F_{1, 177} = 11.95$, $P = 0.0007$), individuals which resulted in a general decrease in average flight bout time compared to fed adults. Effects of nutrition were observed in old *A. auroguttatus* adults ($F_{1, 177} = 46.23$, $P = \leq 0.0001$) which had shorter flight bouts when starved.

Total weight loss. Nutritional status, pre-flight weight and elytron length had a significant effect on total weight loss following the 24 hour flight trial. Fed adults lost significantly more weight after being tethered to flight mills for 24 hours than starved individuals ($F_{1, 178} = 19.05$, $P = \leq 0.0001$). Additionally, *Agrilus auroguttatus* adults with a heavier pre-flight weight and larger body size (elytron length) lost significantly more weight after being tethered to flight mills for 24 hours ($F_{1, 178} = 17.35$, $P = \leq 0.0001$, and $F_{1, 178} = 7.35$, $P = 0.0074$, respectively). Finally, gender, age, and mating status were not significant to post-flight weight loss ($P = \geq 0.05$).

Table 3.1. Average (mean ± SE) flight parameters measured for starved *A. auroguttatus* adults under varying gender, age, and mating status.

Flight Parameter	Starved							
	Male				Female			
	Young		Old		Young		Old	
	Virgin	Mated	Virgin	Mated	Virgin	Mated	Virgin	Mated
Total distance flown (m)	379.85 ± 89.54	270.23 ± 69.87	253.26 ± 122.59	145.81 ± 99.76	926.15 ± 177.85	900.12 ± 198.50	422.14 ±103.70	197.68 ± 79.20
Total flight time (min)	46.74 ± 10.81	40.36 ± 9.17	46.74 ± 25.34	13.30 ± 7.31	80.55 ± 18.49	82.29 ± 15.78	52.29 ± 10.21	33.12 ± 8.22
Ave. flight velocity (m/s)	0.14 ± 0.02	0.13 ± 0.04	0.09 ± 0.03	0.15 ± 0.07	0.27 ± 0.07	0.18 ± 0.03	0.14 ± 0.03	0.08 ± 0.02
No. of flight bouts	126.9 ± 24.7	116.2 ± 23.9	108.9 ± 41.8	35.5 ± 15.6	178.6 ± 36.7	160.3 ± 21.18	178.8 ± 34.9	95.5 ± 22.1
Ave. flight bout time (s)	19.49 ± 2.60	24.72 ±4.05	18.12 ± 2.69	17.49 ± 3.14	25.72 ± 3.22	30.03 ± 4.74	19.02 ± 2.66	95.54 ± 22.13
Elytron length (mm)	6.99 ± 0.18	7.16 ± 0.12	7.16 ± 0.19	7.11 ± 0.18	8.25 ± 0.07	7.74 ± 0.18	8.16 ± 0.14	8.32 ± 0.14
Total weight loss (mg)	2.90 ± 0.81	1.91 ± 0.28	1.90 ± 0.35	2.18 ± 0.40	2.36 ± 0.43	1.83 ± 0.30	2.23 ± 0.26	2.23 ± 0.23
n	10	11	10	11	11	12	13	13

Table 3.2. Average (mean ± SE) flight parameters measured for fed *A. auroguttatus* adults under varying gender, age, and mating status.

Flight Parameter	Fed							
	Male				Female			
	Young		Old		Young		Old	
	Virgin	Mated	Virgin	Mated	Virgin	Mated	Virgin	Mated
Total distance flown (m)	592.15 ± 188.28	654.08 ±158.61	1078.22 ± 240.04	1011.62 ± 185.37	1077.22 ± 475.43	842.23 ± 195.61	1889.46 ± 494.39	1761.26 ± 253.28
Total flight time (min)	59.59 ± 15.60	71.79 ± 16.32	83.55 ± 15.01	81.90 ± 17.50	73.26 ± 30.46	63.96 ± 18.28	84.76 ± 19.72	108.35 ± 18.60
Ave. flight velocity (m/s)	0.23 ± 0.06	0.27 ± 0.08	0.18 ± 0.03	0.30 ± 0.06	0.22 ± 0.04	0.28 ± 0.08	0.41 ± 0.05	0.28 ± 0.03
No. of flight bouts	152.5 ± 30.3	172.0 ± 40.0	169.2 ± 29.7	160.8 ± 30.6	106.9 ± 26.1	109.6 ± 25.1	108.3 ± 17.0	226.2 ± 33.7
Ave. flight bout time (s)	23.97 ± 3.47	29.53 ± 4.11	27.99 ± 4.26	34.35 ± 5.47	33.62 ± 9.56	33.34 ± 6.84	45.77 ± 8.38	75.13 ± 43.52
Elytron length (mm)	7.13 ± 0.23	7.04 ± 0.17	6.98 ± 0.17	6.98 ± 0.22	7.65 ± 0.29	7.65 ± 0.32	7.79 ± 0.14	8.01 ± 0.08
Total weight loss (mg)	2.40 ± 0.34	2.20 ± 0.39	2.64 ± 0.43	2.85 ± 0.30	2.50 ± 0.40	4.02 ± 0.90	3.25 ± 0.39	3.77 ± 0.26
n	10	10	11	13	10	12	12	13

Discussion

Assessing the flight potential of *A. auroguttatus* adults of varying age, mating, and nutritional status using computerized flight mills has provided essential information regarding the dispersal potential of this invasive beetle. The nutritional status and body size (elytron length) of *A. auroguttatus* adults had a significant influence on overall flight performance. The total distance, flight time, velocity, number and duration of flight bouts, and post-flight weight loss was greater in larger, fed individuals when tethered to flight mills and flown for a 24-hr trial period. Since flight is a very energy-intensive activity, the enhanced flight performance of fed adults is not surprising (Thompson and Bennett 1971, Candy et al. 1997). Although elytron length was correlated with overall flight performance, pre-flight weight was not. While an increase in body mass could represent an increase in flight muscle mass, body mass also includes lipid content, water content, and non-flight musculature (Shelton et al. 2006), which may explain its lack of influence on *A. auroguttatus* flight. However, the correlation of pre-flight weight with the total amount of weight lost following 24-hr flight trials suggests that heavier adults utilize more energy resources during flight than lighter individuals.

The influence of mating status and gender was not important to many parameters of *A. auroguttatus* flight including total flight distance, duration, velocity, and flight bout time. In contrast, the flight distance and duration of mated emerald ash borer (*Agrilus planipennis* Fairmaire) females was significantly greater then unmated females or males

when tethered to flight mills, which suggested that females of this species may be programmed for dispersal flights following mating (Taylor et al. 2010). However, the lack of mating and gender effects on the overall flight performance of *A. auroguttatus* adults is similar to that reported for the plum curculio *Conotrachelus nenuphar* (Herbst) (Chen et al. 2006), and the Chinese white pine beetle (*Dendroctonus armandi*) (Chen et al. 2011), respectively.

Significant interactions between nutrition and age were observed in the total flight distance, flight time, velocity, number of flight bouts, and flight bout time. Age had an effect on flight performance when adults were starved, but not when adults were fed, resulting in overall decreased flight activity in old, starved individuals during the 24-hr trial period. These results indicate that age is an important component of dispersal when energy resources are depleting, but does not impact flight when resources are sustained. The reduced flight performance of old *A. auroguttatus* individuals under starvation conditions points to the difficulty for long distance dispersal by this species across habitats that lack suitable oak hosts, especially considering the age of 'old' individuals in this study (typically 6 days old). Old, starved *A. auroguttatus* flew an average of 255 m in 24 hrs. Considering the average dispersal capabilities of these individuals, it would take approximately 6 years to travel from oak woodlands in southern Arizona to San Diego Co. (a distance of approx. 550 km). When the average distance flown by all individuals in this study (approx. 790 m in 24 hrs) is taken into account a dispersal of 550 km would require approximately 2 years of continuous flight. Therefore, it appears

unlikely that *A. auroguttatus* is capable of range expansion by natural dispersal across geographic barriers such as the Sonoran and Mojave deserts, which was hypothesized by Westcott (2005). Similarly, dispersal between mountain range populations of *A. auroguttatus* in southern Arizona, which are also separated by approximately 60-150 km of the Sonoran desert, doesn't seem likely. These results support the hypothesis that *A. auroguttatus* was accidently transported into southern California through infested wood, which is becoming an increasingly important transport vector of woodborers and bark beetles (Liebhold et al. 1995, Perrings et al. 2005, Brockerhoff et al. 2006, Hulme et al. 2008, Tobin et al. 2010).

In summary, our results suggest that nutrition, body size, and the interaction between nutrition and age are important factors for *A. auroguttatus* dispersal. These finding are essential for understanding the overall risk of *A. auroguttatus* in California, and for implementing specific management strategies for this pest including the use of 'trap' or sentinel trees, which are now used in North America to detect and provide a semiquantitative measure of migrating emerald ash borer where dispersal is anticipated (Muirhead et al. 2006). This information can also help to define quarantine zones surrounding infestation sites, which may help to contain *A. auroguttatus* within infested regions, thereby preventing further spread of this invasive beetle.

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Chapter 4

Population Genetics of Goldspotted Oak Borer (Coleoptera: Buprestidae): Investigating the Origin of an Invasive Pest of Native Oaks in California

Abstract

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, is an invasive woodborer that is native to oak woodlands across southern Arizona. Developing a classical biological control program for this pest in southern California, U.S.A. is a high priority due to the continuing ecological and economical damage caused by this insect since its recent introduction into the area. Analyses of the mitochondrial cytochrome oxidase COI and ribosomal nuclear 28SD2 gene regions provide insight into the phylogeographic relationship between and within populations of *A. auroguttatus* in Arizona and California, in an attempt to determine the area of origin for this invasive beetle. The area of origin for the invasive population of goldspotted oak borer in California was not determined conclusively, although our data suggests the Dragoon Mountains in Cochise Co., Arizona as a possible source for the California population of *A. auroguttatus*. Results also confirmed that individuals collected from populations across southern Arizona and California are all *A. auroguttatus*, and are not part of a cryptic species complex parallel to that observed with the morphologically similar *A. coxalis*. Future surveys for natural enemies of *A. auroguttatus* will focus on the Dragoon

Mountains as a potential source for co-evolved enemies for use in a classical biological control program against this invasive woodborer in southern California.

Introduction

The goldspotted oak borer, *Agrilus auroguttatus* Schaeffer, (Coleoptera: Buprestidae) is an invasive wood-boring beetle that aggressively attacks native oak trees in southern California, U.S.A. Native to Arizona, this beetle was initially detected in the Descanso Ranger District, Cleveland National Forest, San Diego County, California, in 2004, but was likely introduced accidentally several years earlier through movement of infested oak firewood (Coleman and Seybold 2008, Coleman et al. 2012a). Infestation of *A. auroguttatus* in southern California currently covers approximately 213,000 ha across San Diego, and Riverside Counties (Jones et al. 2013), and mortality to native Coast live oak, (*Quercus agrifolia*), California black oak (*Q. kelloggii* Newb.), and canyon live oak (*Q. chrysolepis* Liebm) from *A. auroguttatus*, caused by extensive larval feeding damage to the phloem/xylem interface, is estimated at > 22,000 trees (Coleman et al. 2012b). The documentation of oak mortality since 2002 indicates that the distribution of this beetle is radially expanding within San Diego Co. (USDA Forest Health Monitoring [FHM] 2011), which can mostly be attributed to natural dispersal. However, satellite infestations in Marion Bear Memorial Park, San Diego Co. (approximately 32 km from the closest known infestation) in 2009, and in Idyllwild, Riverside Co. (approximately 70 km from the closest known infestation) in 2012 are hypothesized to have resulted from further

movement of infested oak firewood (Jones et al. 2013), indicating that this is a viable dispersal mechanism for *A. auroguttatus* in California.

The introduction and establishment of invasive wood-boring and bark beetles has become increasingly problematic in recent decades (Liebhold et al. 1995, Perrings et al. 2005, Brockerhoff et al. 2006, Hulme et al. 2008, Tobin et al. 2010). Invasive pests such as the emerald ash borer, *Agrilus planipennis*, Asian longhorn beetle, *Anoplophora glabripennis*, and the red turpentine beetle, *Dendroctonus valens*, have caused a tremendous amount of ecological and economical damage after being introduced in non-native habitats through the transportation of infested wood material (Yan et al. 2005, Poland and McCullough 2006, Haack et al. 2010). Managing these invasive species in natural ecosystems is challenging because at the landscape level, the majority of tools available (i.e., chemical or physical/mechanical control) work only if the infested area is small or sufficiently isolated to prevent re-infestation (Van Driesche et al. 2010). Eradication of newly introduced, isolated populations of invasive wood-boring and bark beetles in a forest environment is typically not successful because of the difficulty in detecting these invaders at an early stage. Concealed inside trees or other wood material, these insects are often overlooked until extensive decline and mortality occurs within tree hosts (Coleman and Seybold 2008a, Kovacs et al. 2010). Classical biological control can be a very efficient management strategy for controlling invasive species in natural ecosystems since biological control agents have the ability to become established and

remain in the environment indefinitely, providing long-term and widespread management of the pest without continuous human intervention (Van Driesche et al. 2010).

Molecular markers have the potential to supply valuable information for making management decisions about invasive species by characterizing population level genetic variation, which in turn can aid reconstruction of the history of an invasion, and identify the geographic origin of the invading population (Cognato et al. 2005, Rugman-Jones et al. 2007, Simonsen et al. 2008, Bray et al. 2011). This information can be especially useful when developing a classical biological control program because often little is known about a species prior to its establishment and subsequent invasiveness in a non-native habitat. Insight into the invasion history and source of an invasive pest can help to identify transport vectors and direct the search for co-evolved biological control agents (Rugman-Jones et al. 2007). Understanding genetic variation within and among populations of an invasive species (in both the native and introduced range) can also simply help to clarify taxonomy of a pest. Indeed, molecular analyses reported in Coleman et al. (2012a) already helped to resolve long-running taxonomic confusion between *A. auroguttatus* and its morphologically similar congener *A. coxalis* Waterhouse (Hespenheide et al. 2011), and narrowed the native distribution of *A. auroguttatus* to southern Arizona, and perhaps northern Mexico (based on a single specimen collected from Baja California Sur in 1977) (Hespenheide et al. 2011), though this suspected population has yet to be confirmed (Coleman et al. 2012b).

Within southern Arizona, records indicate that *A. auroguttatus* has been collected from several mountain ranges including the Chiricahua, Huachuca, Santa Catalina, and Santa Rita mountains since the early 1900's (Coleman and Seybold 2011). These mountain ranges can be considered "sky islands", isolated by the surrounding Sonoran desert, which presents a dispersal barrier for *A. auroguttatus* and its natural enemies. The first collection of *A. auroguttatus* from the Dragoon mountains in 2011 suggests that there are additional mountain ranges within the greater home range that may be suitable for *A. auroguttatus* inhabitation, and some of these (e.g., the Whetstone and Patagonia Mountains) have not been systematically surveyed for this beetle. Narrowing the geographic source of the invasive California population to a specific mountain range in Arizona will, in theory, allow the collection of natural enemies which have formed a co-evolutionary relationship with the genotype of the invasive population (Stouthamer 2008).

The mitochondrial cytochrome oxidase (COI) gene has been particularly useful in studies investigating the source of an invasive population (Havill et al. 2006, Rugman-Jones et al. 2007, Cognato et al. 2005). Alternatively, the highly conserved ribosomal nuclear gene 28SD2 has been used to separate species which are morphologically indistinguishable (Rugman-Jones et al. 2010, Coleman et al. 2012a). In the present study, we utilize sequences of the mitochondrial cytochrome oxidase (COI) and 28SD2 genes to investigate connectivity among *A. auroguttatus* populations from the San Jacinto Mountains and Cleveland National Forest in southern California, and the Chiricahua, Dragoon, Huachuca, Santa Catalina, and Santa Rita Mountains in southern Arizona. Our

goal was to better understand genetic variation within and between populations in the native and introduced ranges, with the hope of identifying the source of the invasive California population. This information is a fundamental step in the search for co-evolved natural enemies of *A. auroguttatus* that may be considered for use in a classical biological control program against this pest in southern California.

Materials and Methods

Specimen Collections. Specimens of *A. auroguttatus* were collected between May 2009 and November 2012 from infested oak trees in San Diego and Riverside Counties, California, and across several mountain ranges in southern Arizona, U.S.A. (Table 4.1; Fig. 4.1). Individuals collected in California were either: removed from purple and green prism sticky traps (used to trap flying adults) which were placed throughout the Descanso Ranger District in the Cleveland National Forest, San Diego Co. (DRD-CNF); or reared from infested *Quercus agrifolia* and *Q. kelloggii* material collected from this area (Coleman and Seybold 2011). All life stages of *A. auroguttatus* were also collected by destructively sampling infested *Q. agrifolia* and *Q. kelloggii* trees found in DRD-CNF, and in the town of Idyllwild, in the San Jacinto Mountains, Riverside Co. Arizona specimens were also collected by destructive sampling or reared from infested *Q. emoryi* and *Q. hypoleucoides* (Coleman et al. 2012a). All specimens collected in this study were preserved in 95% ethanol and stored in a -20° freezer until DNA analyses.

Table 4.1. Collection and voucher information for populations of *Agrilus auroguttatus*.

Locality	County	State	GPS coordinates	Collection dates (mo/yr)	Map reference
Chiricahua*	Cochise	AZ	31°50′N 109°17W	1/10	G
Dragoons	Cochise	AZ	31°53′N 109°59′W	5/11	F
Huachuca*	Cochise	AZ	31°24′N 110°18′W	1/10	E
Santa Catalina*	Pima	AZ	32°26′N 110°47′W	1/10	C
Santa Rita*	Santa Cruz	AZ	31°43′N 110°52′W	5/09– 2/12	D
Cleveland National Forest*	San Diego	CA	33°18′N 116°48′W	5/09 – 8/11	A
San Jacinto	Riverside	CA	33°44′N 116°42′W	10/12 – 11/12	B

An asterisk indicates that a representative specimen is sequenced and deposited in Genbank.

Fig. 4.1. Map of collection sites for *A. auroguttatus* in southern California and Arizona. See Table 4.1 for map legend with corresponding collection information.

DNA Extractions. Whole genomic DNA was extracted from individual specimens using a standard Chelex method (Walsh et al. 1991). Individual *A. auroguttatus* were removed from ethanol and allowed to air-dry on filter paper. A tissue sample was then taken from each specimen and transferred to a 0.5 µl microcentrifuge tube containing 4 µl of proteinase-K. Tissue samples comprised either a single hind tibia for adult specimens, or a small slice (approx 1-3 mm^3) from the head capsule of larval specimens. Tissue samples were homogenized in the proteinase-K using a micropestle, after which, 100 µl of a 5% Chelex® 100 (Bio-Rad Laboratories, Hercules, CA) suspension (in water) was added and the tubes were incubated at 55°C for 1 h, then at 99°C for an additional 10 min to inactivate the proteinase-K. The chelex resin and insect debris were pelleted in a microcentrifuge at 14,000 rpm for 4 minutes, and the supernatant (containing the extracted DNA) was transferred to a new 0.5 µl microcentrifuge tube and stored at -20°.

Amplification of Extracted *A. auroguttatus* DNA. Genetic variation across California and Arizona populations of *A. auroguttatus* was examined by amplifying a section of the mitochondrial gene (mtDNA) cytochrome oxidase c subunit 1 (COI) using the polymerase chain reaction (PCR). Reactions were performed in 25 µl volumes containing 2 µl of DNA template (concentration not determined), 1 × ThermoPol PCR Buffer (New England BioLabs, Ipswich, MA, USA), an additional 1 mM $MgCl_2$, 200 µM each of dATP, dCTP, & dGTP, and 400 µM dUTPl, 4% (v/v) BSA (NEB), 1 U *Taq* polymerase (NEB), and 0.2 µM each of the primers LCO1490 (5'-

GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). PCR was performed in a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA) with the following settings: 2 min at 94°C; followed by five cycles of 30 s at 94°C, 1 min 30 s at 45°C, and 1 min at 72°C; followed by a further 35 cycles of 30 s at 94°C, 1 min 30 s at 51°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. A section of the D2 domain of 28S (28S-D2) nuclear ribosomal DNA (rDNA) was also amplified from a subsample of specimens, representing a broad diversity of COI haplotypes (see results) and individuals collected from each locality, using the primers 28sF3663 (5'-TACCGTGAGGGAAA GTTGAA-3') with 28sR4076 (5'-AGACTCCTTGGTCCGTGTTT-3') and protocol described in Rugman-Jones et al. (2010).

Cleaning and Sequencing. Successful amplification was confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide. PCR products were subsequently cleaned using the Wizard® PCR Preps DNA purification system (Promega, Madison, WI, USA) and sequenced in both directions at the University of California Riverside Genomics Institute, Core Instrumentation Facility. Sequences were aligned manually in BioEdit 7.0.5.3 (Hall 1999) and primer sequences were removed prior to analysis. This resulted in an aligned COI matrix of 286 sequences, each 658 bp long, and an aligned 28S matrix of 23 sequences, each 536 bp long.

Genetic Analysis. COI sequences were translated using the EMBOSS-Transeq website (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) to confirm the absence of nuclear pseudogenes (Song et al. 2008). A haplotype (H) network was constructed using TCS version 1.21 with default run settings (Clement 2000). Genetic diversity within and between sampled populations was examined by calculating the average number of pairwise nucleotide differences using Dnasp v. 5.10.1 (Librado and Rozas 2009). Representative COI and 28S sequences were deposited in GenBank (Benson et al. 2008) (accession numbers: JF719839–JF719885).

Results

A section of the COI gene of 286 *A. auroguttatus* individuals (adults and larvae), 147 from Arizona and 139 from California, was sequenced. Among these sequences, a total of 39 haplotypes were identified. Haplotype distribution, abundance, and diversity across sample locations in Arizona and California is shown in Table 4.2. The average number of pairwise nucleotide differences within and between Arizona and California populations is shown in Table 4.3. The majority of haplotypes are grouped into three distinct genetic clusters (Fig. 4.2). The first cluster comprised 17 haplotypes centered on the most common haplotype (H1), with all haplotypes no more than 2 bp distant. The second cluster again comprised 17 haplotypes, this time centered around H8, with 12 of the 16 "satellite" haplotypes again no more than 2bp distant, and the most distant (H14)

being 5bp distant. Clusters 1 and 2 were separated by 6 nucleotide substitutions. The third cluster comprised of three haplotypes (H31, H21 and H9) detected only in the Chiricahua Mountains, AZ; the easternmost part of the sampled range. Of the remaining two haplotypes, H29 fell more or less between clusters 1 and 2, while H25 was relatively distant from all other haplotypes, at 9 bp from its closest genetic neighbor (H31). Despite this apparent genetic structure, there was little evidence of any geographic signal in the major clustering of the haplotypes (Fig. 4.2). That said, there was also little overlap in the geographic distribution of individual haplotypes. Indeed, only 2 haplotypes were shared between CA and AZ, one from each cluster. The most common haplotype (H1) was shared between eighty-two specimens from Arizona (n = 51) and California (n = 31), including specimens collected from the Chiricahua (n = 7), Dragoon (n = 6), Huachuca (n = 5), and Santa Rita Mountains (n = 33) in Arizona, and from the Cleveland National Forest (n = 27) and San Jacinto Mountains (n = 4) in California. However, the second overlapping California and Arizona haplotype (H34) was shared only between individuals collected from the Dragoon Mountains in Arizona (n = 1) and the Cleveland National Forest in California (n = 4). The remaining 37 haplotypes identified were either from specimens collected only in Arizona (n = 23) or only in California (n = 14). Sequences of 28S rDNA were identical for all individuals sampled from the Chiricahua (n = 1), Huachuca (n = 1), Santa Catalina (n = 1), and Santa Rita (n = 6) Mountains in Arizona, and from the Cleveland National Forest (n = 9), and San Jacinto (n = 5) Mountains in California, confirming that individuals collected from these populations are all *A. auroguttatus*.

Table 4.2. Number of haplotypes and haplotype diversity sampled from individuals collected in each locality within Arizona and California.

Locality	County	State	No. of individuals	No. of haplotypes	Haplotype diversity	Map reference
Chiricahua*	Cochise	AZ	20	6	0.7632	G
Dragoons	Cochise	AZ	23	6	0.6840	F
Huachuca*	Cochise	AZ	25	12	0.9167	E
Santa Catalina*	Pima	AZ	10	3	0.7333	C
Santa Rita*	Santa Cruz	AZ	69	9	0.7123	D
Cleveland National Forest*	San Diego	CA	115	15	0.8594	A
San Jacinto	Riverside	CA	24	3	0.3587	B

An asterisk indicates that a representative 28SD2 sequence is deposited in Genbank.

Table 4.3. Variation in the DNA sequence of a 658bp stretch of the mitochondrial COI gene of *A. auroguttatus*. Average number of pairwise nucleotide differences within (diagonal element) and between geographic regions.

	Chiricahua	Dragoons	Huachuca	Santa Catalina	Santa Rita	Cleveland National Forest	San Jacinto
Chiricahua	4.121						
Dragoons	7.286	5.628					
Huachuca	6.126	6.200	5.807				
Santa Catalina	6.050	7.309	5.584	2.933			
Santa Rita	4.785	6.154	4.498	2.881	1.613		
Cleveland National Forest	7.243	6.386	6.416	7.248	6.124	6.451	
San Jacinto	9.508	6.580	8.138	10.625	9.565	6.932	3.243

See Table 4.2 for the sample size and locality information of each population.

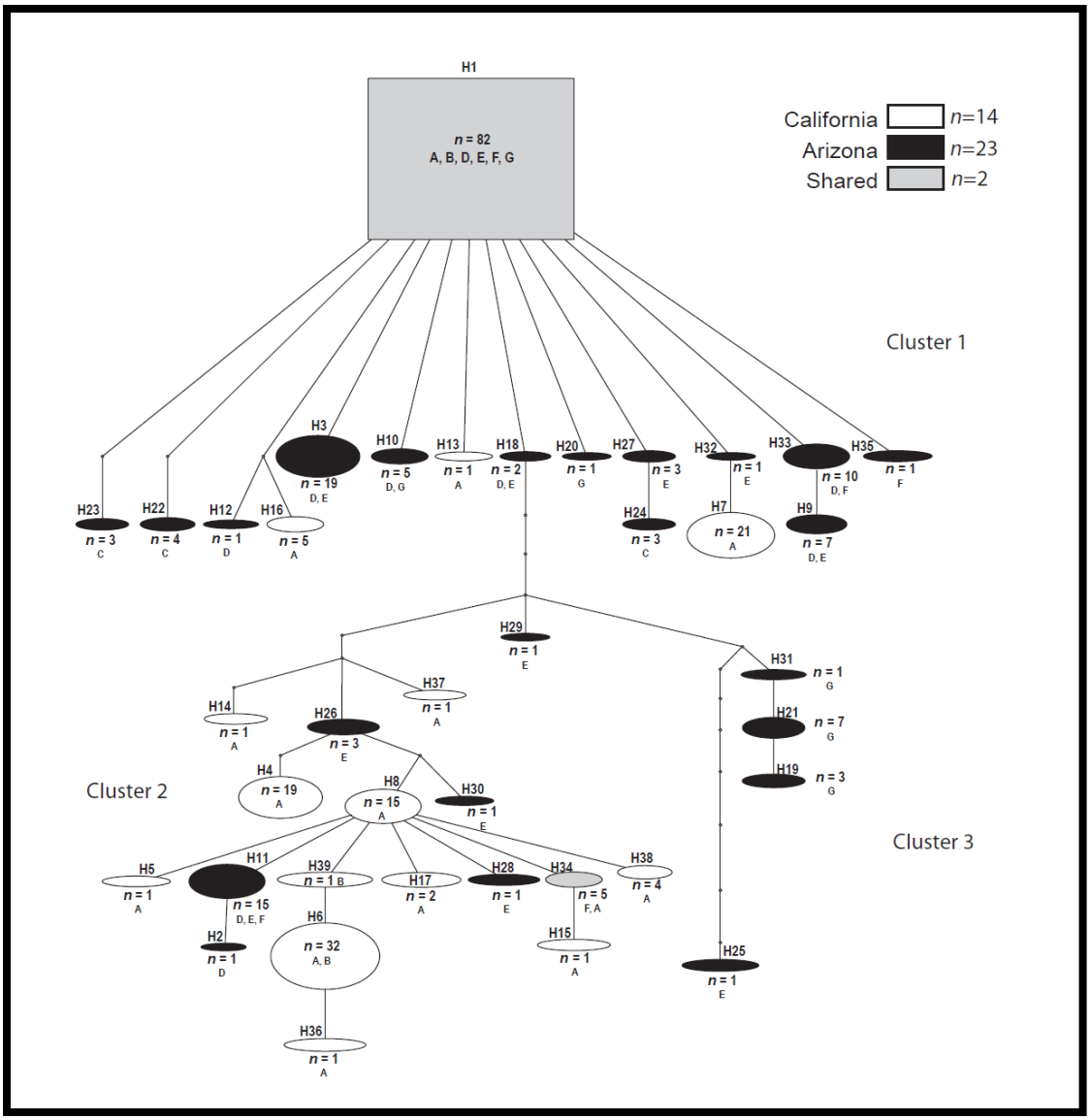

Fig. 4.2. Mitochondrial haplotype network for *A. Auroguttatus* individuals collected in California and Arizona, U.S.A. Each haplotype is represented by a rectangle or oval. Haplotype size is proportional to the number of specimens sharing a haplotype, and the rectangular haplotype is that assigned to the highest outgroup probability. Small circles represent unobserved inferred haplotypes and lines between haplotypes represent a single nucleotide mutational change. Alpha-numeric codes and their corresponding localities are shown in Table 4.1.

Discussion

The recent introduction of *A. auroguttatus* has resulted in mortality to tens of thousands of mature red oaks (section *Lobatae*) in southern California, thereby changing the landscape of important and unique oak woodland communities (Allen-Diaz et al. 2007, Coleman et al 2012b). The difficulty in managing this wood-boring pest in a natural ecosystem has underscored the importance of developing a classical biological control program for *A. auroguttatus* in southern California. Analysis of DNA sequences from the COI and 28SD2 gene regions allowed the comparison of *A. auroguttatus* populations in California and across several mountain ranges in Arizona in an attempt to identify the source of this invasive beetle. The identical nature of 28SD2 sequences from specimens across the sampled geographic range (Table 4.2) indicated that individuals collected in California and Arizona were all one species, and not the cryptic congener *A. coxalis* or perhaps another 'unknown' cryptic species (Coleman et al. 2012a). The identification of 39 haplotypes from 286 individuals across all sample locations showed there was high variability in the COI gene region of this species, but, except for the most abundant haplotype (H1), the distribution of the haplotypes revealed little genetic overlap between the native and introduced regions. That said, of the native populations sampled, the Dragoon Mountains in the Coronado National Forest, Arizona, was the only one to share additional haplotypes with the invasive Californian populations, highlighting it as a likely source of the invasion.

The mixture of California and Arizona haplotypes among the three clusters in Fig. 4.2 provides little evidence of any geographic signal in the major grouping of haplotypes in this network. Surprisingly, 37 of 39 haplotypes sampled across California (*n* = 14) and Arizona (n= 23) populations were distinct to either region. Out of the 23 distinct haplotypes identified from Arizona collections, 17 of those were unique to either the Chiricahua (*n* = 4), Dragoon (*n* = 1), Huachuca (*n* = 7), Santa Catalina (*n* = 3), or Santa Rita (*n* = 2) Mountains, implying a fair amount of genetic isolation between these different populations of *A. auroguttatus* in southern Arizona. In California, 13 distinct haplotypes were identified from collections in the Cleveland National Forest in San Diego Co., and a single haplotype was collected in the San Jacinto Mountains, Riverside Co., California. The relatively high genetic variation observed in the introduced population of *A. auroguttatus* is an unexpected result since a substantial decrease in genetic diversity is often the result of founder events (Slade and Moritz 1998, Gwiazdowski et al. 2006, Havill et al. 2006). However, high genetic variation was also observed in the introduced range of the invasive red turpentine beetle, *Dendroctonus valens*, in China (Cognato et al. 2005), suggesting a single, large introduction, and/or multiple small introductions from different regions in southern Arizona. Furthermore, within our sample, 31 specimens collected from the bark and phloem of a single tree in Arizona yielded six haplotypes (data not shown). Given this high level of diversity in a single tree in Arizona, it is possible that the California population may have arisen from a single geographic source point, and may be from just one infested tree in Arizona.

Two haplotypes were shared between populations in California and Arizona indicating the presence of some genetic similarity between the native and introduced range. The most common haplotype (H1) was shared between eighty-two specimens, and was detected in every population sampled, except the Santa Catalina Mountains in Arizona. The prevalence of this haplotype throughout the native and introduced range does not help delineate the origin of the invasive California population, and is likely to occur in all regions within the native range. However, the second overlapping California and Arizona haplotype (H34) was shared only between individuals collected from the Dragoon Mountains in Arizona (n = 1) and the Cleveland National Forest in California (n = 4). Due to the scant overlap between haplotypes identified from California and Arizona, the shared H34 haplotype may indicate that the Dragoon Mountains in Arizona is the most likely source of the invasive California population, and consequently should be an area of focus when surveying for natural enemies of *A. auroguttatus*. However, it seems unlikely that we have yet sampled the full range of variation in GSOB, and larger samples will be needed to pinpoint the geographic source of the California population of *A. auroguttatus.* Furthermore, we cannot rule out the possibility that the 'true' source of the invasive population currently remains unsampled. One possibility is that *A. auroguttatus* originated from a population in northern Mexico. Little is known of the distribution of *A. auroguttatus* in this region, but it certainly seems plausible to expect that *A. auroguttatus* may occupy similar "sky islands" in the Sonoran Desert as it stretches across the border (see Fig. 4.1). Rigorous, systematic collections from the Chiricahua, Dragoon, Huachuca, Santa Catalina, Patagonia, and Whetstone Mountain

ranges are necessary. It is also feasible that a single variable marker, in this case COI, simply does not contain enough information to accurately predict the source of the invasive GSOB populations, and additional markers may be required. The development and use of microsatellites, or the newly developing field of RAD sequencing may provide a more in-depth comparison of DNA fragments, which may offer a better understanding of the genetic variation in and between *A. auroguttatus* populations, and help to more conclusively determine the area of origin for this invasive pest (Puritz et al. 2012).

The prompt characterization of co-evolved and efficient natural enemies of *A. auroguttatus* in Arizona is a critical component to the overall success of this classical biological control program because over time, the distribution and population densities of this beetle may be too large to feasibly control with this method. Of the potential source populations surveyed, the results of this study suggest the Dragoon Mountains as the most likely invasive source of *A. auroguttatus* in southern California. Surveys for natural enemies in the near future will focus on the Dragoon Mountains.

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Chapter 5

Mortality factors affecting Agrilus auroguttatus Schaeffer (Coleoptera: Buprestidae) eggs in the native and invaded ranges

Abstract

An absence of diverse and coevolved natural enemies may explain the high levels of oak mortality caused by an invasive wood boring beetle, *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae), in California (CA). A field study was conducted to test the enemy release hypothesis for a single guild of natural enemies by comparing mortality factors affecting *A. auroguttatus* sentinel eggs deployed in both native (southern Arizona [AZ]) and introduced ranges (southern CA). The percentage of eggs attacked by natural enemies did not differ between sites, which does not support the enemy release hypothesis for this life stage. Although the predominant cause of mortality to sentinel eggs deployed in CA and AZ was due to factors other than natural enemy activity, chewed, missing, and parasitized eggs contributed to as much as 16% and 24% of sentinel egg mortality in CA and AZ, respectively. In addition, the first known egg parasitoid of *A. auroguttatus* was collected during this study from a single egg deployed in AZ, and was identified as *Trichogramma* sp. using molecular techniques. This parasitoid is a generalist, and therefore not suitable for use in a classical biological control program against *A. auroguttatus* in CA. A continuation of this study is needed across a larger number of field sites and over a longer period of time to optimize the

potential detection of host specific egg parasitoids for potential introduction into CA as part of a future classical biological control program, and to better quantify natural enemy impacts on *A. auroguttatus* eggs.

Introduction

The goldspotted oak borer (GSOB), *Agrilus auroguttatus* Schaeffer (Coleoptera: Buprestidae), is an invasive pest that was accidently introduced into southern California's (CA) oak forests. *Agrilus auroguttatus* is native to southern Arizona (AZ) and was likely introduced into CA through the transportation of infested oak firewood. This beetle was initially detected in San Diego County, CA in 2004 (Westcott, 2005), but was not associated with the area-wide decline and mortality of indigenous oaks until 2008 (Coleman and Seybold, 2008a). Aerial surveys of oak mortality since 2002 suggest that the incipient population of *A. auroguttatus* was introduced into southern CA several years prior to its initial detection in 2004, and is expanding its range (Coleman *et al.*, 2012a).

Unlike most *Agrilus* species which are associated with oak trees already in decline (e.g., *A. bilineatus* [Weber] in the northeastern United States and *A. bigutattus* [Fabricius] in Europe), *A. auroguttatus* is the primary cause of mortality to coast live oak (*Quercus agrifolia* Nèe), California black oak (*Q. kelloggii* Newb.), and canyon live oak (*Q. chrysolepis* Liebm.) in southern CA (Coleman *et al.*, 2012a). Mortality of these native species is estimated at >22,000 trees within the 212, 460 ha infestation zone, and is

caused by extensive larval feeding damage to the phloem/xylem interface (Coleman *et al.*, 2012a). *Agrilus auroguttatus* preferentially attacks the main stem of large diameter (>12 cm at breast height) trees in the red oak group (section *Lobatae*), although minor injury to the white oak, *Quercus engelmanni* Greene (section *Quercus*), has been observed (Coleman and Seybold, 2011). Symptoms of infestation include crown-thinning and dieback, D-shaped exit holes, bark staining, and woodpecker damage (Hishinuma *et al.*, 2011).

The oak forests of southern CA are largely composed of species from the red oak group, which hold dominant and co-dominant positions in the canopy, and are highly vulnerable to *A. auroguttatus* infestation (Coleman and Seybold 2008b). The widespread loss of these foundation species is anticipated to result in detrimental effects to southern California's oak savanna and mixed conifer ecosystems by affecting energy and nutrient inputs, hydrology, food webs, and biodiversity (Ellison *et al.*, 2005). Biodiversity in these unique forests is expected to be negatively impacted due to the loss of habitat and food resources for native wildlife (McShea *et al.*, 2007). Additionally, the accumulation of dead oak litter from trees killed by *A. auroguttatus* will alter the fuel load in affected areas, increasing the probability and severity of wildfires (Coleman and Seybold, 2008b).

Strategies under investigation for *A. auroguttatus* management include topical and systemic insecticide use, wood solarization (i.e., wrapping infested wood in plastic and heating in the sun to kill larvae or pupae in wood, or to trap and kill adults as they

emerge), and grinding infested wood (Coleman and Seybold, 2008a). These strategies are designed for either treating individual, privately owned trees (e.g., insecticides), or for slowing expansion of the infestation zone via the movement of infested firewood (e.g., wood solarizing and grinding). Currently, there is no functional strategy to manage this beetle in a forest environment. While the efficacy of insecticide use for *A. auroguttatus* management is currently being evaluated, pesticide use to protect trees in forest stands is neither cost effective, sustainable, or environmentally appropriate. The development of a classical biological control program for suppressing *A. auroguttatus* populations with co-evolved host specific parasitoids is an appealing forest management strategy in southern CA since it has the potential to be permanent, widespread, cost-effective, and environmentally safe.

Classical biological control has been an effective tool for lowering populations of non-native forest pests (Hajek, 1999; Ryan *et al.*, 1978; Roland and Embree, 1995; Van Driesche *et al.*, 2010). The recent mortality of millions of native ash trees (*Fraxinus* spp.) by the invasive emerald ash borer (*Agrilus planipennis* Fairmaire) in North America has highlighted the destructive capabilities of introduced woodborers on native forest ecosystems. Management of *A. planipennis* in the northeast has focused on biological control since containment through early detection, quarantine, and infested tree removal has had little success (Cappaert *et al.*, 2005; Duan *et al.*, 2011). Egg parasitoids have shown potential as biological control agents of *A. planipennis* (Liu *et al.*, 2007). These natural enemies, should they exist for *A. auroguttatus*, are of high interest for use in the

emerging biological control program for this pest in CA due to the efficiency of producing eggs in the laboratory as compared to other life stages (e.g., larvae), which in the absence of artificial diet, require cut logs for rearing, which are not effective for rearing larvae through to adults.

In its native range in southern AZ, *A. auroguttatus* is not pestiferous and exhibits behavior similar to other *Agrilus* species that preferentially attack trees already in decline (Coleman *et al.*, 2012a). The rarity of *A. auroguttatus* specimen collections from museum and field surveys, and lack of data in the economic entomology literature denotes the relative insignificance of this beetle regarding oak forest health in southern AZ (Coleman and Seybold, 2011). In comparison, the elevated levels of oak decline and mortality in southern CA could be due to the new association of *A. auroguttatus* with ecologically naïve hosts and a lack of host-specific natural enemies in the introduced range (Coleman and Seybold, 2011).

The enemy release hypothesis predicts that alien species introduced into a new region should experience reduced impacts from natural enemies which will lead to an increase in their distribution and abundance (Roy *et al.*, 2011). This hypothesis is the theoretical foundation of classical biological control (Liu and Stiling, 2006), and has been commonly used to explain the success of invasive pests, especially plant and arthropod species, when they are uncoupled from their co-evolved natural enemies (Cincotta *et al.*, 2009; Georgiev *et al.*, 2007; Keane and Crawley, 2002; Koyama and Majerus, 2008).

The disproportionate population densities of *A. auroguttatus* in the introduced range suggest that the success of this woodborer in southern CA could be due, in part, to release from co-evolved natural enemies.

Here, we test the enemy release hypothesis on a single guild of natural enemies by comparing the mortality factors of *A. auroguttatus* sentinel eggs deployed in both native (southern AZ) and introduced ranges (southern CA). This study will help to determine the potential role natural enemies play on the population dynamics of this beetle in CA and AZ. Results from these field surveys with deployed *A. auroguttatus* eggs provide useful data for identifying surveying techniques and potential egg parasitoids for future use in a classical biological control program against *A. auroguttatus* in southern CA.

Materials and methods

Study sites. Field studies were conducted in the native (Arizona [AZ], USA) and introduced range (southern California [CA], USA) of *A. auroguttatus*. Two oak forest field sites, one in each state, were selected from counties where this beetle had been previously collected (Coleman and Seybold, 2011). Site 1 (31°43'N/110°43'W; 1509-1523 m), part of the native range of *A. auroguttatus*, was an approximately 1 hectare plot located at Gardner Canyon in the Santa Rita Mountains, Pima County, AZ, USA. Site 2 (33°02'N/116°35'W; 1277-1296 m), part of the invaded range in southern CA, an approximately 1.4 hectare plot, was located at William Heise County Park, San Diego

County, CA, USA. At each site, six trees (infested with larval *A. auroguttatus*) were selected for deployment of *A. auroguttatus* sentinel eggs to measure mortality factors on this life stage in the native and invaded ranges. Trees were considered infested if symptoms described in Hishinuma *et al.* (2011) such as larval galleries and exit holes were present. At each study tree, sentinel eggs were deployed in non-caged, caged, and exclusion treatments to determine whether egg mortality rates were affected by increasingly limiting natural enemy access to sentinel eggs. The deployment of *A. auroguttatus* eggs at each site was conducted over an 8 week period during July - September 2012.

Preparation of *A. auroguttatus* sentinel egg masses. *Agrilus auroguttatus* sentinel eggs were produced in the laboratory by allowing field-collected adults to deposit their eggs onto coffee filter paper. *Agrilus auroguttatus* adults were reared from infested *Q. agrifolia and Q. kelloggii* trees that were felled in April 2012 at William Heise County Park, Julian, CA, cut into rounds (approximately 30 x 60 cm), and placed inside 15 emergence tents (also located at William Heise County Park). During June to August 2012, adults were collected daily from emergence tents (1.83 x 1.83 x 1.83 m Lumite® screen portable field cages, Bioquip Products, Rancho Dominguez, CA) containing approximately 15 *A. auroguttatus*-infested oak rounds. From these daily collections, a mixture of 10-15 reproductively mature males and females were introduced into 2.13 liter hand-grip rearing containers (11.7 x 12.1 x 18.1 cm, Candy Concepts Inc., Pewaukee, WI) with a 6 cm diameter ventilation hole that was covered with fine metal

mesh screen. Adults that died were replaced with other reproductively mature adults to maintain a consistent number of 10-15 adults in each rearing container. A total of 10 rearing containers were held under ambient laboratory conditions (14:10 L:D, 24 ± 4°C, 35 ± 5 % RH) and contained host plant leaves (*Q. kelloggii*), moist cotton wick as a water source, and standard white coffee filter paper (11.1 cm diameter base, Ambiance™, Amerifoods Trading Co., Los Angeles, CA) as an oviposition substrate. The coffee filter paper was cut into approximately 10 cm diameter rounds and placed directly underneath the rearing container lid. A metal mesh screen was placed on top of the filter paper which held the paper in place, secured the ventilation hole, and provided adults with a textured substrate for gripping while ovipositing. This method of acquiring eggs has been used successfully for research on emerald ash borer (*A. planipennis*) biological control (Yang *et al.*, 2012). Coffee filters were placed inside each rearing container for 1-2 days, removed, and quarter rounds with eggs were excised for placement onto cut oak branches (see below for more details on egg deployment). Sectioned coffee filters with eggs were numbered, eggs were counted before field deployment, and were 2-3 days old at the time of field placement.

Field deployment of sentinel *A. auroguttatus* egg masses. *Agrilus auroguttatus* eggs on sectioned and labeled coffee filters were attached to cut oak branches. Cut oak branches were collected in the field by cutting branches (approximately 6 cm in diameter x 18 cm in length) from native AZ (*Q. emoryi*) and CA oaks (*Q. agrifolia*) using a pruning saw. Egg-infested oak branches were made by attaching two quarter round egg

papers (these were numbered and the number of eggs deployed per treatment were recorded) to an oak branch with nickel plated thumbtacks. An eye-loop bolt was screwed into the top of each cut oak branch for hanging (approximately 12 cm from trunk) on hooks that were attached to the trunks of selected trees.

Oak branches with *A. auroguttatus* eggs were randomly assigned to one of three treatments: (1) non-caged branches, where *A. auroguttatus* eggs were fully exposed to natural enemies (parasitoids and predators), (2) caged branches, where branches were suspended inside metal mesh cylinders to exclude large generalist predators, but preferentially allow parasitoid access, and (3) exclusion cages that used the same cage from (2) above, which were fully enclosed within a fine mesh bag to exclude all natural enemies. All three treatment types were individually suspended on each of six *A. auroguttatus*-infested trees, for a total of 18 egg-infested oak branches deployed at each field site.

Wire mesh cages used in the caged treatment were made by forming a tube (approximately 24 cm length & 15 cm diameter) out of 0.3 cm hardware cloth and securing with zip-ties. At the top and bottom of each tube, a flat square of hardware cloth (approximately 12 x12 cm) was attached using zip-ties, thereby enclosing the tube with an *A. auroguttatus* egg-infested oak branch inside. Egg-infested oak branches were held inside each cage using an eye-loop bolt that was screwed in from the outside of the cage, through an opening in the mesh of the top square of hardware cloth, then into the egg-

infested oak branch. The bottom square of hardware cloth was attached after an egg-infested oak branch was in place. Egg-infested oak branches were positioned in the center of the cage so that no contact was made between the branch and cage walls.

The exclusion treatment was made by placing a caged treatment into an "exclusion bag". Exclusion bags (approximately 45 cm in length x 30 cm in diameter) were made by sewing white no-see-um netting (lightweight no-see-um fine polyester netting, approximately 100 holes per sq. cm, Skeeta, Bradenton, FL). Exclusion bags were closed with a drawstring thereby fully enclosing the hardware cloth cage. Each treatment type was hung from the eye-loop bolt onto a selected tree using a screw-in ladder hook (19 cm length x 4.5 cm height, Crawford® Ladder Hook (Ss11-50), Lehigh Consumer Products, LLC, Rye, NY) and polypropylene rope. Ladder hooks were screwed approximately 1.5 m above the ground into the trunk of a selected *A. auroguttatus*-infested tree. For each treatment type, a flat white sticky trap (19.5 x 16 cm) was hung through the rope connecting the eye-loop bolt to the ladder hook and was situated above the suspended oak branch bearing the *A. auroguttatus* egg papers. Sticky traps were used to discourage potential egg predation by ants.

Retrieval and rearing of *A. auroguttatus* sentinel egg masses. Sentinel eggs were deployed for 7 days and replaced on this weekly schedule at both study sites for 8 consecutive weeks. Eggs on each egg paper were counted immediately after collection to determine the number of missing eggs per replicate for each treatment. Egg papers

collected from field sites were placed immediately into sterile 100 mm x 15 mm polystyrene Petri dishes, sealed with Parafilm M® (Peniney Plastic, Chicago, IL), and transported under valid permit to the Insectary and Quarantine facility at the University of California, Riverside. Egg papers were stored under ambient laboratory conditions (14:10 L:D, 23 ± 2 °C, 30% RH) and checked every other day for three weeks for parasitoid emergence. Three weeks was considered a sufficient time period for egg incubation following field collections because under laboratory conditions (14:10 L:D, 23 ± 2 °C, 30% RH), *A. auroguttatus* neonate larvae emerge 10-15 days after oviposition (VML, unpublished data).

Three weeks post-collection, eggs on each piece of coffee filter paper were examined under a dissecting microscope and assigned to one of 6 categories: 1) hatched - a visible *A. auroguttatus* emergence hole, 2) unhatched - no visible emergence hole, 3) non-viable - no melanization and shriveled, 4) chewed - fragments of egg chorion remaining on paper, 5) parasitized - visible parasitoid with associated exit hole or parasitoid life stage inside the egg, or 6) missing - no egg or chorion remaining on papers. All unhatched eggs were dissected under a stereomicroscope to determine whether under-developed *A. auroguttatus* larvae or parasitoids were present. If unhatched or hatched insects could not be identified by morphology, DNA was extracted from individuals and analyzed.

DNA extraction and analysis of parasitoids collected from *A. auroguttatus* eggs. DNA was extracted from unidentified individuals collected from *A. auroguttatus* eggs using the EDNA HiSpEx tissue kit (Saturn Biotech, Perth, Australia), following the manufacturer's protocol for 1 mm^3 of tissue, but reducing the volume of each kit component to one quarter of that suggested, thereby resulting in a final 25 µL extraction. DNA isolation using this kit involves simple mixing of three proprietary solutions, no grinding of the specimen, and incubation at 95 °C for 30 min. Two adult parasitoids that emerged from *A. auroguttatus* eggs collected in AZ were tentatively identified as Trichogrammatidae by morphology. Therefore, PCR was performed on these and 5 unidentified pre-pupae found inside blackened, unhatched *A. auroguttatus* eggs also collected in AZ using the "ITS2-forward" and "ITS2rev-Trich" primers according to the protocol developed by Stouthamer *et al.* (1999). Representative sequences were deposited in GenBank (Benson *et al.*, 2008).

Statistical analyses. Percentage egg mortality for each category was calculated based on the total number of eggs deployed each week onto individual egg-infested branches (Duan *et al.* 2011). Effects of site (two levels) and treatment (three levels) were analyzed using nested ANOVA. Because the species identity of the six trees selected at the CA site differed from those at the AZ site, a nested ANOVA was used with the level of tree nested under the level of site. The percentage of unhatched, non-viable, and missing eggs did not meet normality assumptions, and were transformed and analyzed on a natural logarithm scale. The percentage of chewed eggs was analyzed using the

Cochran-Mantel-Haenszel test since data transformation was insufficient to meet normality assumptions. Finally, Fisher's exact test was used to determine whether the combinations of site and treatments were independent of percentage parasitization. All statistical analyses were conducted at the 0.05 level of significance and were performed using SAS 9.2 (SAS Institute Inc., 2008).

Results

The fates of *A. auroguttatus* sentinel eggs deployed in Gardner Canyon, Pima County, AZ, and William Heise County Park, San Diego County, CA are shown in Table 5.1 and 5.2, respectively. The majority of eggs deployed in AZ and CA hatched (56-72%), while 9-18% were non-viable, and 9-13% simply did not hatch. Unlike healthy eggs which are rounded, smooth, and begin to melanize several hours following oviposition, non-viable eggs were easily distinguished from healthy eggs by their shriveled, sunken appearance, and lack of melanization. Non-viability was observed before and after AZ and CA egg deployment, and was most likely due to infertility or possibly egg damage during oviposition. During stereoscope inspection, fully formed first instar larvae were observed inside unhatched eggs that were deployed in the AZ and CA sites. Unsuccessful hatching of eggs may be attributed to environmental conditions such as unfavorable temperature and/or humidity either in the field or laboratory. The percentage of missing (3-23%) and parasitized (0.2%) eggs was greater in AZ than in CA (2-14% and 0%, respectively), while the percentage of chewed eggs was marginally

greater in CA (0.5-2%) than in AZ (0.1-1%). Missing eggs were considered to be the result of predation by natural enemies (e.g., predators that could fly and land directly onto egg papers) since *A. auroguttatus* eggs that are oviposited onto coffee filter paper do not easily fall off, even during handling in windy and rainy conditions.

Table 5.1. Fates of *Agrilus auroguttatus* sentinel eggs deployed at Gardner Canyon, Pima County Arizona.

Fate of deployed eggs	Non-caged	Caged	Exclusion
Total no. of eggs deployed	3237	2554	1637
No. hatched eggs (%)	1850 (57.2)	1683 (65.9)	1132 (69.2)
No. parasitized eggs (%)	7 (0.2)	0 (0)	0 (0)
No. unhatched eggs (%)	304 (9.4)	295 (11.6)	162 (9.9)
No. non-viable eggs (%)	301 (9.3)	408 (16.0)	294 (18.0)
No. missing eggs (%)	745 (23.0)	148 (5.8)	48 (2.9)
No. chewed eggs (%)	30 (1.0)	20 (0.8)	1 (0.1)

Table 5.2. Fate of *Agrilus auroguttatus* sentinel eggs deployed at William Heise County Park, San Diego California.

Fate of deployed eggs	Non-caged	Caged	Exclusion
Total no. of eggs deployed	2492	1991	1193
No. hatched eggs (%)	1713 (68.7)	1106 (55.5)	863 (72.3)
No. parasitized eggs (%)	0 (0.0)	0 (0.0)	0 (0.0)
No. unhatched eggs (%)	329 (13.2)	246 (12.4)	119 (10.0)
No. non-viable eggs (%)	378 (15.2)	328 (16.5)	187 (15.7)
No. missing eggs (%)	60 (2.4)	281 (14.1)	24 (2.0)
No. chewed eggs (%)	12 (0.5)	31 (1.6)	0 (0.0)

Parasitoid identification from genetic analyses. Parasitism was only observed from a single egg paper that was deployed into a non-caged treatment in AZ. Following DNA extraction and amplification, sequences of the ITS2 gene region identified all seven parasitoids as identical (GenBank accession KC512817). A BLAST search (Zhang *et.*

al., 2000) identified the parasitoids as a *Trichogramma* sp. with greater than 99% certainty. Comparison to a privately held database of *Trichogramma* ITS2 sequences (Richard Stouthamer, University of California, Riverside, unpublished data) produced a 100% match with a currently undescribed *Trichogramma* sp. that had previously been collected from unidentified Lepidoptera host eggs found in date palms (*Phoenix* sp.) and *Eriogonum* sp. in the Coachella Valley, Riverside County, CA.

Comparison of egg deployment treatments. There was no significant difference ($P \geq 0.05$) between treatment type or site for the percentage of hatched and unhatched eggs deployed in AZ and CA. The percentage of non-viable eggs was significantly different between non-cage and exclusion treatments deployed in both CA and AZ ($F_{2,20} = 3.69$, $P = 0.04$), with exclusion treatments having a higher percentage of non-viable eggs. Additionally, the percentage of non-viable eggs was significantly greater in the CA site than in the AZ site ($F_{1,10} = 6.11$, $P = 0.03$). The percentage of missing eggs between treatments was significantly greater in non-cage treatments than in exclusion treatments ($F_{2,20} = 5.33$, $P = 0.01$), while site effects were only marginally significant ($F_{1,10} = 4.79$, $P = 0.05$). Pair-wise treatment contrasts on the percentage of chewed eggs showed a significant difference between cage and exclusion treatments ($\chi^2 = 12.31$, df = 1, $P = 0.0005$), with cage treatments having the highest percentage of chewed eggs. The percentage of chewed eggs was also significantly greater in non-cage treatments than in exclusion treatments ($\chi^2 = 9.79$, df = 1, $P = 0.002$). However, there was no significant difference in the percentage of chewed eggs between sites. Fisher's

exact test showed a strong association in the percentage of parasitization for the combination of site and treatments ($P \leq 0.0001$). This can be attributed to the single collection of parasitized eggs from one egg card deployed in a non-caged treatment in AZ. Finally, there was no significant difference ($P \geq 0.05$) between treatments or site for the percentage of eggs damaged by natural enemies (no. of chewed eggs + no. of missing eggs + no. of parasitoids found / total no. of eggs deployed) deployed during this study.

Discussion

Understanding the mechanisms behind the contrasting effects of *A. auroguttatus* on oak survivorship in its native and introduced range has been a primary goal in several *A. auroguttatus* research programs (Coleman and Seybold, 2008b; Coleman and Seybold, 2011, Coleman *et al.*, 2011; Coleman *et al.*, 2012a; Coleman *et al.*, 2012b). Hypotheses explaining high levels of tree injury and mortality by *A. auroguttatus* in CA include an absence of diverse and co-evolved natural enemies, and variation in host resistance within the native and introduced range (Coleman and Seybold, 2011). The invasiveness of *A. auroguttatus* in CA is likely attributed to a combination of natural enemy release, low host resistance, and potentially other unknown factors (i.e., high host suitability). However, given the limited number of studies, it is difficult to determine the extent of each of these individual factors, alone or in combination, and their effect on the invasion success of *A. auroguttatus* in CA.

Although initial natural enemy surveys focusing on larval and pupal parasitoids had detected greater species richness and abundance in AZ than in CA (Coleman *et al.*, 2012b), this was not observed in this study for eggs, as the combination of overall factors affecting egg mortality from natural enemies (i.e., missing, chewed, and parasitized eggs) did not differ between locations. This study was conducted over a relatively short, eight week survey period from a single site in both AZ and CA which could have contributed to our low detection of egg parasitoids, just one *Trichogramma* sp., which was recovered from just seven *A. auroguttatus* eggs in AZ.

These results indicate that although predation (i.e., chewed and missing eggs) and parasitism of GSOB eggs was minor compared to other mortality factors (i.e., non-viable and unhatched eggs), natural enemy activity may be an important influence on egg-stage population densities. In AZ, as much as 24% of sentinel egg mortality (from chewed, missing, and parasitized eggs) was contributed to natural enemies. In CA, egg mortality from natural enemies was lower, accounting for 16% of all mortality factors. While no egg parasitoids of *A. auroguttatus* were detected in CA during this study, an unidentified psocopteran was observed inside a chewed *A. auroguttatus* egg. Although most psocids are herbivores or detritivores, a few are partial predators that consume insect eggs and possibly scale insects (Baz, 2008). An in depth study examining egg-stage mortality factors over several generations and sites would help to determine the extent of these predator impacts on *A. auroguttatus* populations in both the native and introduced range.

Importantly, the first known egg parasitoid of *A. auroguttatus* was collected during this study. Prior to this work, egg parasitoids of *A. auroguttatus* were unknown. The parasitoid collected was identified as *Trichogramma* sp., and was obtained from sentinel eggs deployed in the native range. Investigation into the identity of this species (using ITS2 sequences) found a previous collection record of this parasitoid from Lepidoptera eggs collected in Riverside County, California (Richard Stouthamer, University of California, Riverside, pers. comm.). The low percentage of parasitism by this parasitoid, and its previous collection records from non-Coleoptera hosts indicates that this species is likely a generalist that opportunistically parasitized sentinel GSOB eggs in AZ.

The detection of very few egg parasitoids in this study could be the result of inadequate surveying techniques, an insufficient search range or duration, or simply a lack of this particular guild of natural enemies in the native and introduced range. Egg parasitoids of *Agrilus* spp. can be very challenging to locate due to their small size and the concealed locations of their host's eggs, which are often laid under loose bark or in crevices of bark (Duan *et al.*, 2012). *Agrilus auroguttatus* eggs are small (approximately 1 mm in width), turn a brownish color 2-3 days after oviposition, and are laid deep inside the cracks and crevices of oak bark (Lopez and Hoddle, 2013). Additionally, the preferred oak hosts of *A. auroguttatus* have dark, rough, hard bark that does not easily flake away, which makes locating *A. auroguttatus* eggs in the field very difficult. Since 2008, *A. auroguttatus* eggs have only been detected in the field on a single occasion in

which a hatchet was required to remove bark pieces for inspection with a hand lens. This process was time consuming, labor intensive, and had relatively little success. In the laboratory, detection of *A. auroguttatus* eggs oviposited onto bark pieces (approximately 12 x 8 cm) was also challenging due to the cryptic coloration and placement of the eggs deep inside crevices and cracks, and required a stereoscope and dissecting tools for identification. Since the overall structure (e.g., topography, thickness, and coloration) of oak bark from *A. auroguttatus* hosts makes surveying for egg parasitoids by collecting naturally deposited *A. auroguttatus* eggs an arduous task, our strategy of finding egg parasitoids using sentinel egg masses on filter paper is a practical though semi-artificial alternative.

The detection of *A. auroguttatus* egg parasitoids may also benefit from an increased search range and/or duration, especially in the native range in AZ. Our inability to detect *A. auroguttatus* egg parasitization in the introduced range of southern CA tentatively supports the enemy release hypothesis, and was not surprising, even considering the relatively short, eight week study period conducted from a single field site. Similarly, surveys for potential egg parasitoids of *A. planipennis* in its introduced range from 2003 to the present have yet to identify egg parasitoids that are indigenous to North America (Bauer *et al.*, 2008; Liu *et al.*, 2007). However, we did expect to detect higher rates of parasitization in the native range of *A. auroguttatus*. The very low percentage of *A. auroguttatus* parasitization in AZ (0.2%) is minute compared to the >60% parasitization

of *A. planipennis* eggs reported from its native range in China (Liu *et al.* 2007), and could be the result of a more intensive search for *A. planipennis* egg parasitoids.

The *Trichogramma* sp. that was recovered from our study is not suitable for use in a classical biological control program against *A. auroguttatus* in CA due to its lack of host specificity, and its likely presence within infested areas in CA (this parasitoid has been previously recovered from desert areas in southern CA). The identification and utilization of egg parasitoids for the biological control of invasive wood borers such as *A. planipennis* and *Phoracantha semipunctata* (F.) (Coleoptera: Cerambycidae) have shown positive results (Duan *et at.*, 2012; Hanks *et al.*, 1996; Liu *et al.*, 2007), and encourages further surveys for host specific egg parasitoids of *A. auroguttatus*. Specifically, we are interested in locating and identifying encyrtid parasitoids (Hymenoptera: Encyrtidae) of *A. auroguttatus*, should they exist. These parasitoids have been successfully used in the biological control of *A. planipennis*, *A. anixius*, and *P. semipunctata*, and are known parasitoids of several other *Agrilus* species (Duan et al., 2012; Hanks *et al.*, 1996; Muilenburg and Herms 2012; Zhang *et al.*, 2005). In addition, the highest rates of *Agrilus* egg parasitism (>50%) occurred with four species of encyrtid that were reported in North America, Asia, and Europe (Taylor *et al.*, 2012). A comprehensive list of encyrtid egg parasitoids and their *Agrilus* hosts is presented in Taylor *et al.* (2012).

In order to maximize the potential detection of host specific egg parasitoids, and determine the potential impacts of natural enemies attacking *A. auroguttatus* eggs in AZ

and CA, a continuation of this study is needed across a larger number of field sites and over a longer period of time. Methods for conducting future egg parasitoid surveys will factor in results of this study which show non-cage and cage treatments as equally suitable for detecting and acquiring natural enemies of *A. auroguttatus* eggs.

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