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# DNA barcoding helps establish a novel host-plant association for *Chrysobothris mali* Horn, 1886 (Coleoptera: Buprestidae) larvae found infesting *Pyrus communis* L. 'Bartlett' (Rosaceae) fruit in Lake County, California orchards

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*Abstract.* In August 2023, larvae of an unknown buprestid species were found feeding in numerous pear fruits in four orchards in Lake County, California. Larvae were collected and placed on an artificial diet to allow development into adults. A larval sample sent to the California Department of Food and Agriculture was identified as a *Chrysobothris* species (Coleoptera: Buprestidae) based on morphological characteristics. Nondestructive DNA extraction and CoxI barcoding from four larvae identified the buprestid as the Pacific flatheaded borer, *Chrysobothris mali* Horn, 1886, a result later confirmed by morphological identification of reared adults. The Pacific flatheaded borer has a wide host range of at least 70 plant species belonging to 40 genera in 21 plant families. This is the first reported detection of a buprestid attacking fruit, indicating a **new larval host record** and a potential behavioral shift by this species.

*Keywords.* European pear, new larval host, Pacific flatheaded borer

## Introduction

Members of Buprestidae are commonly referred to as flatheaded woodborers as larvae and metallic woodboring or jewel beetles as adults. This family of elateriform beetles is diverse, comprising at least 14,757 described valid species (Bellamy 2008) occurring on all continents, except Antarctica. The genus *Chrysobothris* includes 702 species that are distributed worldwide (Bellamy 2008), among which are at least 63 species that occur in California. One behavioral trait most buprestids have in common is that their larvae are mostly wood boring, primarily associated with trunks and branches of trees and feeding upon the phloem (vascular bundles) or xylem of the plants. Female buprestids typically select stressed or dying plants to lay their eggs on, although females of some species lay eggs on healthy hosts. Larval feeding results

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in a disruption of water and nutrient movement in host plants that causes wilting, dieback, or even death, especially if the trunk is girdled (Nowak et al. 2001, Evans et al. 2004).

Most buprestids are not considered to be pests, but a few are regarded as pest species due to infesting economically important agricultural or horticultural crops resulting in economic damage and loss. Detection of buprestid infestation in host plant species relies upon several available sampling tools and techniques, including: (1) scouting for visual signs and symptoms associated with the presence of larval infestation and adult beetle activity; (2) use of girdled trap trees; (3) subsampling protocols to detect larvae under the bark; (4) artificial traps baited with pheromone and/or host volatile lures; (5) biosurveillance using buprestid hunting wasps; and (6) remote sensing techniques (Ryall 2015). Indirect detection of insects using molecular methods has been employed with heavy reliance upon the 640–658-base pair (bp) 5' portion of the cytochrome oxidase I mitochondrial gene (CoxI) (Folmer et al. 1994, Hebert et al. 2003).

Increasingly, identification of insects has become a synthetic approach employing both traditional and molecular methods. Buprestid larvae have defining characters that can be used for species level identification, including limited numbers of *Chrysobothris* spp., as illustrated in works from eastern Canada (Benoit 1964), central Europe (Bily 1999) and Iraq (Jalil & Ali 2021); yet, buprestid larvae have not been well studied in North America, resulting in a lack of larval keys that can differentiate North American species, including those of commonly encountered *Chrysobothris* spp. that frequently share the same distributional ranges. As such, when utilizing traditional methods focused on morphological characters, adult specimens of buprestids are required. In U.S. agricultural systems, when immature stages cause economic damage to a crop, there are few to no resources available for quick identification of the species. Access to adult specimens requires collecting larvae from infested host plants and rearing until adult emergence. Both options may require weeks to months and specialized effort to meet rearing condition and resource needs leading to unpredictable adult recovery success (Duan et al. 2021). DNA extraction methods to recovery molecular markers like CoxI can improve diagnostic success and decrease time to identification (e.g., Yanase et al. 2013) and can provide identities for damaged and partial specimens recovered following extraction from woody tissues. DNA extraction typically involves destruction of specimens or parts thereof that can result in loss of access to morphological structures essential for species level visual identification (Castalanelli et al. 2010). Nondestructive approaches for DNA extraction involving use of proteinase K in addition to surfactants in the lysis step to help break down structural proteins are becoming commonplace (Martoni et al. 2019, Kirse et al. 2023). These approaches expand opportunities to use archival specimens from myriad repository types and specimens borrowed from private entomological collections. Carew et al. (2018) and Kirse et al. (2023) compared traditional destructive versus nondestructive DNA extractions on bulk arthropod samples, and each found the two methods yielded similar diversity metrics through metabarcoding. Direct molecular identification of any taxon is heavily reliant upon availability of a densely sampled and well curated barcode library. Currently for insects, CoxI is the only gene for which such a marker exists and that has been shown to enable separation of highly similar taxa (Meyer & Paulay 2005). Wu et al. (2017) used CoxI barcodes to compliment adult rearing to successfully identify wood borers, including 16 species of buprestids representing four genera, from solid wood packing intercepted at U.S. ports of entry.

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In August 2023, flatheaded borer larvae of an unknown species were found feeding in numerous 'Bartlett' European pear fruits (*Pyrus communis* L. 'Bartlett') (Rosaceae) in four orchards in Lake County, California. A thorough literature review failed to reveal any previously documented reports of buprestid larvae infesting fruit of any kind, either in natural or commercial settings.

Many *Chrysobothris* species are univoltine and overwinter in their reproductive hosts as larvae, pupate in spring, and emerge as adults in summer. Pear fruits ripen between July and August in Lake County, California, after which the fruit will begin to rot if left under natural environmental conditions. Thus, fruit decomposition would be expected to yield a very different environment than the woody plant tissue in which a woodboring larvae would typically overwinter. For this reason, it was hypothesized that pear fruit would be an unsuitable environment for larvae to complete their life cycle and emerge as adults. Observations of field collected pear fruits infested with flatheaded borer larvae were conducted to determine if pears were suitable hosts to complete the flatheaded borer life cycle.

## Methods and Materials

*Specimen Collection, Preservation, and Archival.* Buprestid larvae were found in four orchards (one conventional and three organic) in 2023 and collected from infested pear fruits in Kelseyville, California, Lake County (39°0′19.4″N, 122°51′0.1″W). Degree of infestation varied between orchards from a conventional orchard where only one flatheaded borer larva was found in an edge tree to 100+ distributed randomly in an organic orchard and collected across a ~3-week period prior to harvest.

Multiple samples of larvae were excised from fruit and preserved in 95% molecular grade ethanol. The first sample was sent to the California Department of Food and Agriculture, Plant Pest Diagnostics Center and identified by Alexey Tishechkin, Senior Biosystematist/Curator of Coleoptera, as a *Chrysobothris* species based upon morphological characteristics. To determine the identity of the species involved, we implemented a combination of traditional taxonomy and CoxI barcoding. Eight larvae were reared to adults on an artificial diet, and one male specimen was sent to The University of Tennessee-Knoxville (UTK) to be identified using morphological characters (Fisher 1942). A subsample of larvae was sent to UTK, and four larvae were selected for CoxI barcoding after nondestructive DNA extraction.

Voucher specimens are deposited at the Essig Museum of Entomology, University of California-Berkeley, with the following catalog numbers: EMEC933632 (*C. mali* larvae in EtOH) and EMEC933633, EMEC933634, and EMEC933635 (three adult *C. mali*).

*Insect Rearing.* Forty-two buprestid larvae were dissected from field-collected pear fruits and individually transferred to a 60-mm petri dish containing artificial diet (Gindin et al. 2009) with the modification of 15 g of dried pear woodchips used for the cortex. The artificial diet was replaced every two weeks. Petri dishes were placed under LED grow lights (GooingTop, HY-021; Shenzhen, Guongdong, China) on a flat desk surface and kept at indoor ambient temperatures. In early November 2023 (~6 weeks post autumnal equinox) petri dishes were sealed with parafilm and placed in a refrigerator set to 7.8 ± 1.2 °C to mimic winter. After 10–12 weeks, petri dishes with live larvae were removed from the refrigerator and placed on a 25.4-cm × 52.7-cm seedling heating mat (VIVOSUN, VS-330101, Ontario, California) set to 28.9 ± 1.1 °C on a flat desk surface under LED grow lights.

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Nine infested pear fruits were placed in a plastic jar cut on the sides and sealed with a screen to facilitate air circulation and prevent insects from entering or escaping. The jar was placed in a work shed with no temperature control near a window to receive partial sunlight. The infested pear fruits were monitored weekly from September 2023 to July 2024. The ambient temperature ranged from 1.4 °C to 39.7 °C.

*DNA Extraction.* DNA was extracted using a silica spin column purification method preceded by nondestructive digestion of tissues within a Tris-buffered lysis solution containing surfactants and proteinase K, consistent with similar solutions commonly used in organic solvent/salting out protocols (Miller et al. 1988). The lysis buffer is comprised of the following components and concentrations: Tris base (50 mM, pH = 8.0; Teknova, Hollister, California); ethylenediaminetetraacetic acid (EDTA) (50 mM, pH = 8.0; (Promega Corp., Madison, Wisconsin); sodium dodecyl sulfate (SDS) (2%; Teknova); sodium chloride (NaCl) (100 mM; Fisher Chemical, Fort Lawn, New Jersey); Tween-20® (0.5%; EM Science, Gibbstown, New Jersey); Triton-X® (0.5%; EM Science).

The distal abdominal segment of each larva was removed, and the thoracic cuticle pierced with an insect pin to enhance lysis buffer access to tissues. Both pieces of carcass were placed into individual 2.0-ml Eppendorf tubes containing 300 μL of lysis buffer and 15 μL of proteinase K (of 20 mg/ml; Omega Biotek, Norcross, Georgia). Tubes were incubated in a dry heat block set between 52 and 55 °C for at least 8 hours. Prior to the addition of lysate, unused PuroSPIN MICRO Silica Spin Columns (Luna Nanotech, Ontario, Canada) were treated to ensure maximal binding affinity (e.g., Zhou et al. 2018). Reconditioning involved adding 125 μL of 1 N HCl (FisherSci, Atlanta, Georgia) to the column, immediate centrifugation for 1 min at 13,000× g, and discarding the eluent. Next, 125 μL of lysis buffer was added to the column, immediate centrifugation for 1 min at 13,000× g, and discarding of the eluent. To each extraction tube, 500 μL of a binding buffer solution (J. K. Moulton, unpublished) containing guanidine thiocyanate (GITC, Fisher Bioreagents, Ottawa, Canada), sodium perchlorate monohydrate ($NaClO_4$) (Thermo Fisher Scientific, Ward Hill, Massachusetts), and absolute isopropanol (Fisher Bioreagents) was added. The sample was inverted several times to ensure mixing before the entire volume was transferred to the reconditioned silica spin column housed in a 2-mL collection tube. The columns were centrifuged for 30 sec at 8,000× g, after which the eluent was discarded. Next, the column was washed twice with 200–300 μL of a wash buffer solution (10 mM Tris, pH = 8.0, 80% ethanol) via centrifugation at 8,000× g for 30 sec, after which the eluent was removed and the column dried via centrifugation at 13,000× g for 1 min.

Elution of DNA from the silica column involved placing the column in a fresh 7-ml microcentrifuge tube, addition of 50 μL of heated 55 °C elution buffer (10 mM Tris; pH = 8.0–8.5) directly to the silica membrane, letting the columns stand for at least 1 min, then centrifugation at 13,000× g for 30 sec. DNA was quantified using a Qubit 2.0 fluorometer, with dsDNA High Sensitivity assay (BioDynami, Huntsville, Alabama) following the manufacturer's suggested protocol.

*Primer Design, Amplification Steps, and Sequencing Protocol.* Amplification was completed via PCR using custom primers (FWD; 5′ – ACA AAY CAY AAR GAT ATY GG – 3′: REV; 5′ – GCN CCR AAN TGY TCY TTY TT – 3′) designed to yield a 782-bp fragment (= 742 bp of data) of CoxI completely encompassing the Folmer et al. (1994) fragment (Hebert et al. 2003) region of 709 bp (= 658 bp of data) produced by primers LCO1490: 5′ – GGT CAA CAA ATC ATA AAG ATA TTG G – 3′ and

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HC02198: 5′ – TAA ACT TCA GGG TGA CCA AAA AAT CA – 3′. Both forward primers share the same 3′ terminus, but our downstream reverse primer adds an additional 58 bp of data. PCR amplifications were performed in a 30-μL reaction volume containing 8 μL of molecular grade water, 15 μL of 2× High Yield PCR Master Mix (Blue Dye) (Syd Labs, Hopkinton, Massachusetts), 3 μL of each primer, and 1 μL of purified larval DNA. PCR cycling parameters were as follows: initial denaturation at 95 °C for 90 sec, followed by five cycles of 96 °C for 15 sec, 53 °C for 20 sec, and 72 °C for 45 sec; five cycles of 96 °C for 15 sec, 50 °C for 20 sec, and 72 °C for 45 sec, 15 cycles of 96 °C for 15 sec, 47 °C for 20 sec, and 72 °C for 45 sec, and 40 cycles of 96 °C for 15 sec, 50 °C for 20 sec, and 72 °C for 45 sec. PCR products were visualized and excised from a 1% agarose gel and sequenced using the PCR primers. Amplicons were cleaned using a silica spin column (Epoch Life Science, Sugar Land, Texas) and in house Qiagen solutions (Openwetware.org). Both DNA strands were cycle sequenced in 1/20$^{th}$ reactions using the PCR primers with BigDye® v3.1 Terminators (FisherSci, Pittsburgh, Pennsylvania). Sanger products were cleaned using Centri-Sep G-50 Sephadex columns (Princeton Separations, Monmouth Junction, New Jersey), dried in a CentriVap Concentrator (Labconco, Kansas City, Kansas), and sent to Brigham Young University for fragment analysis and basecalling using an ABI™ 3730XL DNA Analyzer (FisherSci, Pittsburgh). Resultant chromatograms were reconciled using Sequencher v5.4 (Gene Codes Corp., Ann Arbor, Michigan) and published consensus sequences blasted against the NCBI GenBank database.

*Survey.* The initial discovery of buprestids in pear fruit occurred ~4 weeks before the 2023 harvest which, due to lack of time, prevented determining how widespread larvae in pear fruit were countywide. In August 2024, visual surveys for presence/absence were conducted on foot covering a subset of orchards in three pear growing regions of Lake County, California: ~7.81 hectares in Upper Lake, ~9.63 hectares in Scott's Valley, and ~28.1 hectares in Kelseyville.

## Results

DNA recovery from the four larvae analyzed ranged from 512 to 880 ng and purified amplicons ranged from 582 to 984 ng (Table 1). BLAST searches using generated consensus sequences as queries against sequences within the NCBI GenBank database yielded matches of 97–99% identity across the overlapping 658-bp portion to a representative of *Chrysobothris mali* Horn, 1886, the Pacific flatheaded borer (Table 2) (GenBank Accession: KX283168.1; Voucher RGF16610).

Dissections of numerous infested pear fruits revealed a larval tunneling path from the calyx to the sunny side of the fruit. Although chorions were not found, a blackened

Table 1. DNA concentrations achieved from individual larval specimen samples extracted from infested pear fruit.

| Lab sample code | DNA Quantity (ng) | Amplicon Quantity (ng) |
|---|---|---|
| 368 | 512 | 582 |
| 369 | 548 | 738 |
| 370 | 760 | 984 |
| 371 | 880 | 846 |

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Table 2. Sequenced larval BLAST results recovered from NCBI GenBank.

| Sample (lab code) | Identities (sequence homology %) | Resulting sequence ID via NCBI |
|---|---|---|
| 368 (CK2635) | 620/638 (97%) | KX283168.1 |
| 369 (CK2591) | 622/638 (97%) | KX283168.1 |
| 370 (CK1248) | 629/638 (99%) | KX283168.1 |
| 371 (CK3260) | 630/638 (99%) | KX283168.1 |

spot near the calyx, indicative of damage to the exocarp, supports the supposition that eggs are most often laid in the calyx where the larva enters and begins to tunnel through the pear flesh (Fig. 1). Flatheaded borer larval infestation of the fruit is diagnosed by presence of a small, blackened spot near the entrance point of the calyx and a larger, gradually expanding and hardening blackened spot that appears on the sunny side of the fruit (Fig. 2). The larva remains just under the outer fruit skin and

Figure 1. Larvae enter the pear through the calyx (at the bottom of the fruit) and tunnel through the pear flesh to the sunny side of the fruit.

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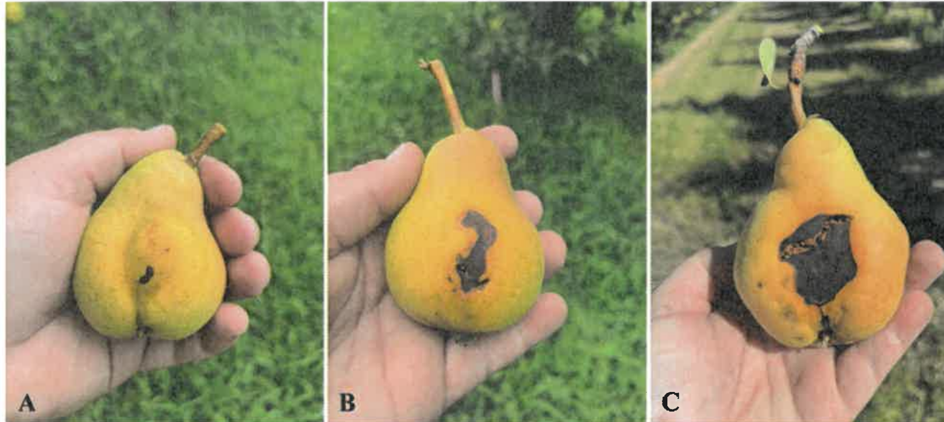

Figure 2. The presence of the flatheaded borer larvae in pear fruit is noticeable by a small, blackened spot near the calyx and a larger blackened spot that begins to appear on the sunny side of the fruit (A) which grows larger (B) and hardens over time (C).

creates a dry pocket that becomes filled with frass and thin leathery layers of pear flesh (Fig. 3). Between one to five larvae were found in each infested pear.

Eight of the 42 larvae taken from infested fruits and allowed to complete development on artificial diet in the laboratory emerged as adults (Fig. 4). The first adult male was sent to The University of Tennessee-Knoxville for morphological identification. Morphological examination of the specimen corroborated results of the previous larval molecular identification. In addition, one adult *C. mali* emerged from the nine overwintered infested pear fruits in late June 2024 (Fig. 5).

Figure 3. The larva creates a dry pocket filled with frass (A) and leathery layers of pear flesh (B) indicated by the arrow.

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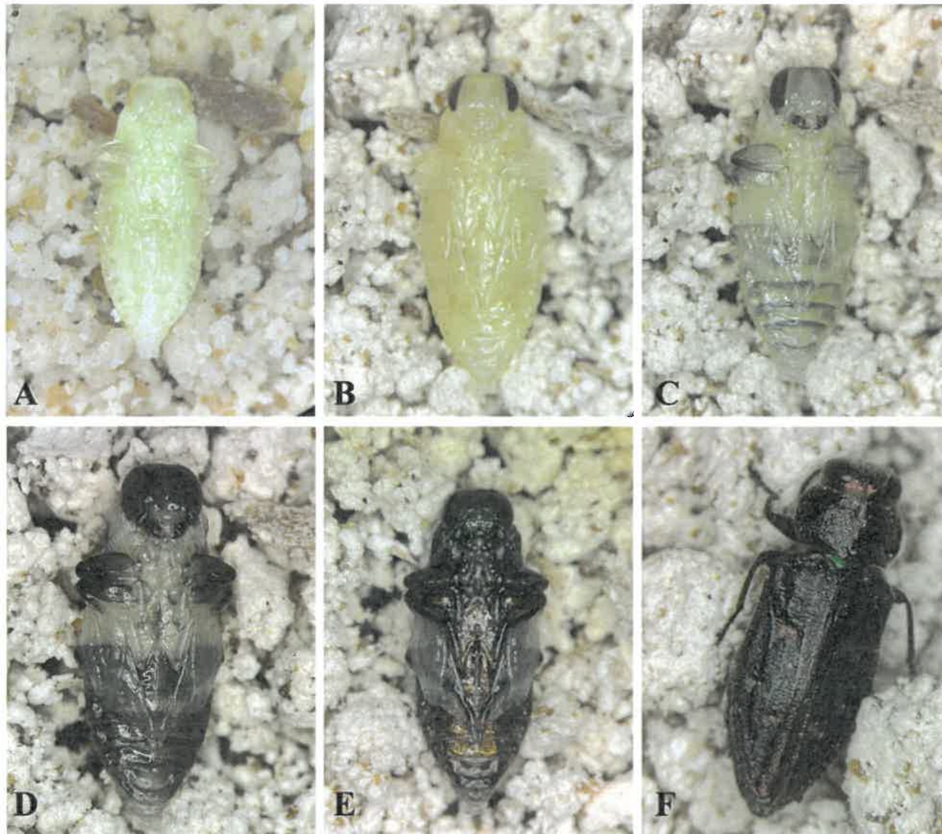


Figure 4. Pupal maturation (A–E) across time showing sclerotization and exoskeleton formation with appearance of metallic coloration after 6 days of observation and adult male *Chrysobothris mali* (F) reared on artificial diet from larva recovered from infested pear fruit.

No flatheaded borer damage to pear fruits were found in the 17.4 hectares surveyed in Upper Lake and Scott's Valley pear growing regions. Flatheaded borer damage to pear fruit in 2024 was found in five organic orchard blocks within a 1.0-km radius of the epicenter (39°0′14.3′′N, 122°50′24.4′′W) in Kelseyville, California.

## Discussion

*Chrysobothris mali*, commonly known as the Pacific flatheaded borer, can be a serious pest in the western United States in addition to a few midwestern states and as far east as Manitoba in Canada, where it attacks many species of shrubs and trees (Acheampong et al. 2016, Rudolph & Wiman 2023). Larvae kill or weaken trees by girdling the trunks and lower branches. Newly planted nursery stock, drought stressed trees, sunburned trees and trees whose trunks are exposed to the sun usually are the most seriously affected. *Chrysobothris mali* attacks the wood of important agricultural commodity crops such as apple, apricot, cherry, peach, pear, plum, prune, walnut, and hazelnut (Burke & *Böving* 1929, Solomon 1995, Nelson et al. 2008, Acheampong et al. 2016, Wiman et al. 2019, Rudolph & Wiman 2023, Homan 2024).

The DNA extraction method used for the identification of *C. mali* larvae collected from infested pear fruit proved to be effective. Considering that DNA extraction

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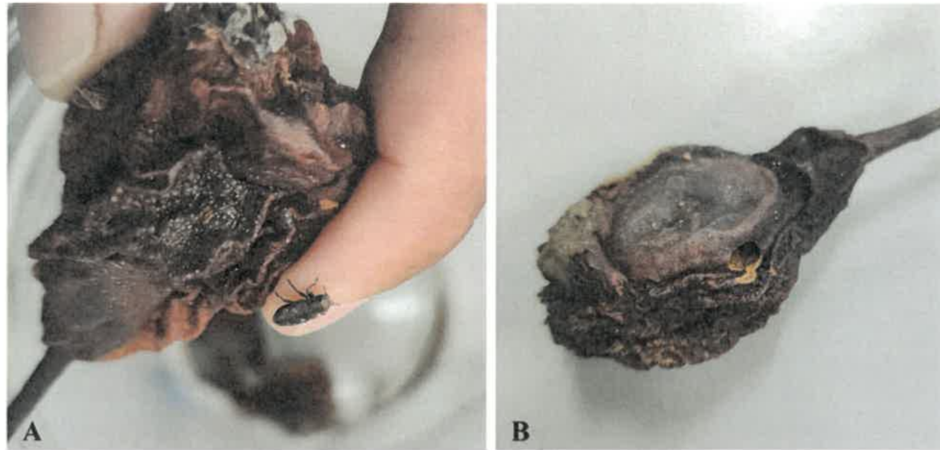

Figure 5. Adult *Chrysobothris mali* (A) reared from overwintering infested pear fruit with exit hole (B).

protocols generally are optimized for dealing with chitinized cuticle of adult specimens (Martoni et al. 2019), the structural integrity of our specimens were preserved, yielding vouchers that could be re-examined and scored in future efforts to develop identification resources, including morphological character illustrations and images for larvae of *Chrysobothris* species (e.g., Bílý 1999, Jalil & Ali 2021). Our modified CoxI primer set captured a slightly larger amplicon (+124 bp) than the conventional > 658-bp one generated by the Folmer et al. (1994) primer set. Use of full length barcodes increases resolving power of BLAST comparisons against database reference sequences in the identification step. This approach is important for pest management as it allows quick identification of potential threats from woodboring species when only immature stages, lacking known morphological characters, are present. These results will also help in documenting behavioral shifts to parts of hosts that were not suspected to be affected by borers.

*Chrysobothris mali* larvae were found in pear fruit in 2023 and 2024, demonstrating use of the fruit as a larval host is a repeated anomaly. Only one *C. mali* larva has been found in fruit on the edge of a conventional orchard, while all remaining detections were found randomly throughout organic orchards. This occurrence can plausibly be explained in that edge trees of an orchard often receive insufficient coverage of pesticides when the spray rig is turning around. Although not all orchards were surveyed, initial results suggest *C. mali* attacking pear fruit appear to be localized to organic orchards within a 1.0-km radius of the original site where the phenomenon was observed (39°0′14.3′′N, 122°50′24.4′′W). More extensive surveying of pear orchards is needed to confirm any association with organic orchards.

One adult emerged from the nine infested pear fruits, resulting in rejecting the hypothesis that pear fruit are not a suitable host for *C. mali* larvae to complete their life cycle. The results demonstrate the dry pocket filled with frass created by larvae is an acceptable microenvironment for larval and pupal development. The field implications of this observation are that adult *C. mali* may be capable of emerging from infested pear fruits that are not harvested and remain in the orchard over winter. Further research is needed to verify if larvae can survive the winter orchard environment and quantify the rate of adult emergence from infested pear fruit in the orchard.

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Further investigation is needed into the differences between pesticides applied in conventional versus organic pear orchards in Lake County. In addition, exploration into any differences in products used or timing of applications between organic orchards with *C. mali* in pear fruit and organic orchards that do not. Future DNA studies are also needed to look for genetic differences between *C. mali* found in pear fruit and *C. mali* from other woody hosts in California to explore possible changes in genetics that could explain the change in larval host behavior. There continues to be a need for further study of the morphological characters of larvae that can be used to identify the buprestid species of North America which are severely understudied.

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