[The Great Lakes Entomologist](https://scholar.valpo.edu/tgle)

Volume 48
Numbers 1 & 2 - Spring/Summer 2015 *Numbers 1 & 2 - Spring/Summer 2015*

Article 3

April 2015

Survival and Phenology of *Agrilus Planipennis* (Coleoptera: Buprestidae) Reared on a Newly Developed Artificial Diet Free of Host Material

Melody A. Keena
USDA

Hannah Nadel
USDA

Juli Gould
USDA

Follow this and additional works at: https://scholar.valpo.edu/tgle

Part of the Entomology Commons

Recommended Citation

Keena, Melody A.; Nadel, Hannah; and Gould, Juli 2015. "Survival and Phenology of *Agrilus Planipennis* (Coleoptera: Buprestidae) Reared on a Newly Developed Artificial Diet Free of Host Material," *The Great Lakes Entomologist*, vol 48 (1)
Available at: https://scholar.valpo.edu/tgle/vol48/iss1/3

This Peer-Review Article is brought to you for free and open access by the Department of Biology at ValpoScholar. It has been accepted for inclusion in The Great Lakes Entomologist by an authorized administrator of ValpoScholar. For more information, please contact a ValpoScholar staff member at scholar@valpo.edu.

Survival and Phenology of *Agrilus planipennis* (Coleoptera: Buprestidae) Reared on a Newly Developed Artificial Diet Free of Host Material

Melody A. Keena[1*], Hannah Nadel[2], and Juli Gould[2]

Abstract

The final phase in the development of an artificial diet that contains no ash host material and the phenology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Bupresidae) on that diet are documented. A diet containing powdered ash phloem exists, but host material introduces potential variability and contamination, and the cost and effort needed to collect and process it can be high. The post-embryonic development of *A. planipennis* was evaluated on four artificial diets lacking host material, and effects of variations in diet layer thickness and moisture content were also investigated. The best diet and rearing method resulted in 67.8% survival to pupation and 51% to adult. Larval size and development rate were comparable to published accounts for emerald ash borer larvae developing on susceptible host plants. Important advances include reduction of antimicrobial components to the lowest functional level; change of protein sources from wheat germ, brewer's yeast, and casein to soy flour and casein; reduction of diet moisture content to 50%; and adding a fresh layer of diet to spent diet half-way through larval development. The artificial diet represents a step toward the development of a standardized mass-production system for *A. planipennis*.

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Bupresidae), is a native of Asia that was found in metropolitan Detroit, Michigan in July 2002 and shortly thereafter in Windsor, Ontario, Canada. It threatens ash trees in urban landscapes and natural forests throughout much of the United States (Haack et al. 2002, Poland and McCullough 2006). Infestations resulting from movement of infested nursery stock or firewood and adult emerald ash borer dispersal contributed to its spread and it is now under federal quarantine in parts of 24 states and two Canadian provinces (USDA-APHIS 2014). All ash trees native to eastern United States are susceptible to emerald ash borer, which is capable of killing a tree within 1–4 years if a sufficient number of larvae feed in the secondary phloem and outer xylem to girdle the tree (Poland and McCullough 2006, McCullough et al. 2009). A recent economic analysis suggests that annual costs associated with emerald ash borer are about $1.7 billion

[1]Northern Research Station, Forest Service, United States Department of Agriculture – 51 Mill Pond Road, Hamden, CT 06492, USA.
[2]Center for Plant Health, Science and Technology, Animal and Plant Health Inspection Service, United States Department of Agriculture – 1398 West Truck Road, Buzzards Bay, MA 02542, USA.
*Corresponding author: (e-mail: mkeena@fs.fed.us).

Disclaimer: The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any product or service to the exclusion of others that may be suitable.

(2014 dollars) in combined government expenditures and value lost to private land owners (Aukema et al. 2011). Early efforts to eradicate emerald ash borer populations were not successful and chemical control can be used to protect high value urban trees but is impractical in forests (Herms and McCullough 2014). Biological control using parasitoids and introduction of resistance traits to susceptible native ash species through breeding have promise as cost-effective and environmentally safe, albeit long-term, alternatives for control of emerald ash borer (Cappaert et al. 2005, Poland and McCullough 2006, Bauer et al. 2008, Rebek et al. 2008, Herms et al. 2009, Whitehill et al. 2012).

A reliable, year-round source of large numbers of laboratory-reared emerald ash borer would advance biological control efforts and promote studies of control strategies that require laboratory bioassay. An artificial diet containing powdered ash phloem was developed to rear larvae to the adult stage, but is limited by time-consuming phloem harvesting and preparation, and inconsistent larval survival (Gould et al. 2004). Mass production may be hampered by potential variability introduced through host material in the diet and susceptibility to microbial contamination. Larval parasitoids of emerald ash borer are currently reared on emerald ash borer larvae reared in small ash bolts and the egg parasitoids are reared on eggs produced by either reared or wild caught adults (Duan et al. 2012, USDA 2013). Preliminary trials indicate that the larval parasitoid *Spathius agrili* Yang (Hymenoptera: Braconidae) will parasitize emerald ash borer in the artificial diet described in this paper (H. Nadel, personal observation), which could eliminate the need for ash products in rearing larval parasitoids if biology is not altered in the process. Research focusing on understanding mechanisms of ash resistance to emerald ash borer in Asian ash species requires a standardized bioassay, without variability introduced by host material, to evaluate resistance-related phytochemicals and proteins (Cipollini et al. 2011; Whitehill et al. 2011, 2012). Efforts to screen pesticides are equally restricted by the resources needed to obtain large numbers of even-aged individuals from infested wood. The current method for obtaining emerald ash borer adults and larvae from infested wood is laborious, puts constraints on lab resources and time, and is inadequate for many practical research applications. Year-round mass rearing would greatly facilitate research and management of emerald ash borer.

Several semi-artificial diets have been developed for rearing coleopteran wood pests but little information exists on artificial diets for rearing Buprestidae. Gould et al. (2004) reported some success rearing emerald ash borer on an artificial diet developed originally for the weevil *Hylobius transversovittatus* (Goeze) (Blossey et al. 2000) with ash phloem powder substituted for the original host-plant material. Gindin et al. (2009) reported on a successful diet for *Capnodis* spp. that contained cortex tissue from the host plant. To date, all artificial diets on which buprestid larvae can complete development contain some host plant material except a diet for the buprestid *Capnodis tenebrionis* (L.) that allowed some larvae to complete development (egg to adult) in 130 d, but percentage mortality data are lacking (Mourikis and Vasilaina-Alexopoulou 1975).

The diets tested in this paper were formulated using methodology similar to diet development for a cerambycid, *Anoplophora glabripennis* (Motschulsky) (Keena 2005). Information was incorporated from published diets for buprestids that contain host material, a study of the nutritional composition of an earlier formulation of the diet compared with phloem from various *Fraxinus* species (Hill et al. 2012), and general information on insect nutritional requirements. The initial approach was to modify existing coleopteran diets, some of which were previously tested on buprestid larvae, by substituting cellulose for host-plant material and replacing some ingredients with others more readily available. However, almost no larvae survived. The next phase evaluated systematic modifications of individual ingredients and additions of others (resulting in 20 different diets), which did little to improve larval survival rates. Subsequently,

the diets were further modified (15 additional diets, Supplemental Table 1) to evaluate various quantities of antimicrobial compounds and sources/quantities of protein on larval survival (nutritional analysis indicated the diet contained more protein than the amount present in susceptible ash phloem). Physical modifications to the rearing system were also assessed during this phase, including infestation with eggs or neonates, vertical or horizontal orientation of rearing dishes, and ways to replace or replenish spent diet. During this phase, reduction of protein content and modification of the protein source (without other changes) did not significantly improve survival and development on the artificial diet (Supplemental Table 2). However, a reduction of antimicrobials and a modified orientation of the rearing dish from horizontal to vertical improved larval survival and development, some even surviving from egg to adult (Supplemental Table 2). At each step in the process, new modifications were compared with the best diet and technique available.

This paper documents the final phase in the development of an artificial diet that contains no host plant material (*Fraxinus* spp.), and the phenology and survival of emerald ash borer on that diet. Three experiments are presented. The first compares larval survival and development on four diet formulations, each presented to the larvae in thin or thick layers of diet. The second compares larval survival and development on a diet containing three moisture levels. The third documents larval size, weight, and instar in destructively sampled cohorts reared on the best diet over time. The best diet and rearing methods have since been used to rear six generations of emerald ash borer.

Materials and Methods

Diet production and preparation. Artificial diets were made in a 12-L steam-jacketed kettle. Ingredients are given in Table 1 in the order in which they were added. All work surfaces and the kettle were disinfected before the diet was made, and workers wore disposable gloves and lab coats to minimize diet contamination. The diet was continuously mixed with a paddle assembly from the time the heat was turned on until the diet was ready to pour. In the first step, the starting base was added to the kettle and brought to a rolling boil. Second, dry mix #1 was added to the kettle. Third, following integration of dry mix #1, dry mix #2 and the wet mix were added and the mixture was allowed to return to a rolling boil for 30 seconds before the heat was turned off, after which the diet continued to boil for several minutes. This was necessary to kill bacteria and denature any enzymes, especially in the wheat germ, that could degrade the diet. Fourth, after the diet was mixed without heat for 5 minutes and allowed to cool, the vitamins and fiber were added. When the last ingredient was thoroughly mixed in (about 10 minutes after the heat was turned off), the mixer was turned off. The diet was covered with plastic film to prevent microbial contamination and medium desiccation while aliquots were processed.

The diet was rolled into 3 or 6 mm thick sheets. Two wooden or Plexiglas platforms (5 × 46 cm, 3 or 6 mm high) were taped to the countertop 30 cm apart and covered with a sheet of heavy duty aluminum foil taped at the corners after it was smoothed over the platforms. About 240–480 g (depending on desired thickness of the sheet) of the hot, unsolidified diet were shaped into a long cylinder and placed on the foil mid-way between the two platforms with the long axis parallel to them. A sheet of heavy waxed paper was put over the diet and a heavy metal rod was rolled over the platforms to flatten the diet into sheets as thick as the platforms (3 or 6 mm). The diet, still between the foil and waxed paper sheet, was removed to cool on a rack and a new foil bottom and wax paper top were used to roll out the next diet sheet. Once the diet cooled to room temperature, the foil and wax paper were removed and the diet sheets were placed individually in 0.049 mm thick resealable plastic bags (Uline.com, S-10824); up to five bagged single sheets were placed inside 0.098 mm thick resealable

Table 1. Diets evaluated for rearing *Agrilus planipennis*. Pre-mixed groups of ingredients are presented in sequence of addition during diet preparation.

		Diet and amount (g)			
	Ingredient (vendor, item #)	**1**	**2**	**3**	**4**
Starting Base	Distilled water	5,000	5,000	5,000	5,000
	Agar *Gracilaria* species (mooragar.com, 41005)	220	220	220	220
	Sodium bicarbonate (grocery store, baking soda)	5	5	5	25
Dry Mix #1	Casein (Americancasein.com, AC-130)	200	200	200	200
	Sucrose (grocery store, table sugar)	300	300	300	300
	Wesson salt mix without Fe (testdiet.com, 25671)	45	45	45	45
	Sorbic acid (insectrearing.com, 6967)	5	5	5	5
	Calcium propionate (sigmaaldrich.com, 18104)	5	5	5	5
	Methyl paraben (insectrearing.com, 7685)	5	5	5	5
	Potato starch (nuts.com)	200	200	200	200
Dry Mix #2	Wheat germ (insectrearing.com, 1659)	500	300	0	300
	Soybean flour (insectrearing.com, 1500)	0	200	500	200
Wet Mix	Cholesterol (insectrearing.com, 5180)	19	19	19	19
	Wheat germ oil (insectrearing.com, G5960)	24	24	24	24
Vitamins & Fiber	Choline chloride (insectrearing.com, 6105)	4	4	4	4
	Vitamin A acetate beadlets (ZMC-USA.com, 325 CWS/GFB)	5	5	5	5
	Vitamin mix (insectrearing.com, F8128)	71	71	71	71
	Cellulose (insectrearing.com, 3425)	1,500	1,500	1,500	1,500

Vendor website and product number are provided in parentheses after each ingredient, when possible.

plastic bags (Uline.com, S-7457). Bagged sheets were held at 10 °C or room temperature until moisture content was analyzed on a sample from each sheet.

Freshly made diet sheets contained about 60% moisture (the driest the diet can be made and still be manipulated before it solidifies), which was determined to be excessive for rearing (MA Keena, unpublished data), so the sheets were laid on a plastic rack (raised 9 cm above the bench surface) to dry in a laminar flow hood before use. The hood was first thoroughly cleaned and sterilized with UV lights for 30 minutes to minimize microbial contamination during drying. Depending on the relative humidity in the room, the diet required 6–9 hours to dry to the desired moisture content. Diet sheets were turned over every 3 hours to ensure even drying. A 2.5 cm diameter sample of the diet (8–10 cm from the edge) was taken before the drying process began and then again after 4–6 hours to monitor moisture content. In experiment 1, diet samples were weighed before and after drying under a hood for several days (until weight loss stabilized) to calculate moisture content, while in experiment 2, a moisture analyzer (A&D Company Limited, Tokyo Japan, MF-50 Moisture Analyzer) was used. The moisture analyzer was set to dry in standard mode at 200 °C

and stop when the sample lost less than 0.20% weight per minute. Additional interim samples, if needed, and a final sample were taken to confirm moisture content. Once the diet was dried to the desired moisture content, it was stored in a clean resealable plastic bag at ≤ 10 °C until use.

Diet was transferred to rearing dishes under a laminar flow hood sterilized with UV light. Bags containing dried diet sheets were cut open in the hood and used as work surfaces. The bottoms of sterile, air-tight Petri dishes (Falcon™ 50 mm diameter × 9 mm deep) were pressed into the diet to cut out diet disks, and the disks were pressed into the dishes to remove all air pockets between the diet and the bottom of the dish. A symmetric lens- shaped notch (~ 5 mm W × 15 mm L) was cut away from the diet edge to provide an egg infestation chamber. Prepared rearing dishes were labeled with diet batch, sheet number, and moisture content of the dried diet. Diet in dishes was stored up to one month in plastic bags at 10 °C until use.

Experimental insects. Wood naturally infested with immature emerald ash borer was collected in Michigan by either United States Forest Service or Animal and Plant Health Inspection Service personnel, held in refrigeration units (4 °C) for various periods and transferred to room temperature in horizontal cardboard tubes with a clear collection cup attached to one end. Adult emerald ash borer were collected from the cups daily during the week and housed in Petri dishes with fresh ash leaves. The dishes were shipped in Tyvek envelopes under permit in a small cooler to the United States Forest Service quarantine facility in Ansonia, CT. Voucher specimens of adults were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

Beetles were sexed and placed in single-sex, 7.6 L containers (Rubbermaid® 8 quart 22.2 cm L × 21.1 cm W × 22.2 cm H) for a minimum of 6 days of maturation feeding prior to mating in single pairs in 0.9 L Ball ® wide mouth glass jars with a mesh lid or in groups in 7.6 L plastic containers. The lid of the 7.6 L containers had an 11 cm diameter window and was snapped on over a piece of fine mesh cloth. There were also two 5 cm diameter mesh windows on the sides of the containers. Beetles were supplied with fully expanded and hardened foliage of *Fraxinus uhdei* (Wenzig) Lingelsh and water. *Fraxinus uhdei* is an evergreen ash from Mexico that emerald ash borer readily utilizes and its foliage can be harvested year round if maintained in a greenhouse under long day-length lighting. The foliage was harvested in the greenhouse from 2.5 m tall potted trees and gently washed with a dilute soap solution to remove unwanted arthropods, rinsed twice with cool tap water, and allowed to air dry. Drinking water (tap water) was supplied in a 5 cm long wick of 1 cm diameter braided cotton roll (Richmond-dentistry.com, 201208) pushed through a hole in the lid of a 30 ml squat plastic container (Solo® translucent soufflé portion cup, cup P100 and lid PL1). The leaf petiole was either placed in the water container beside the cotton roll (single pairs) or through a hole in the lid of a 236 ml plastic cup (group matings) (berryplastics.com, cup T30908CP and lid L309). A paper towel was placed in the bottom of the container to absorb excess moisture. The egg-laying substrate was built with a 20 cm long × 1.9 cm diameter solid PVC rod (single pairs) or a 23.5 cm × 4.8 cm diameter PVC pipe (group matings) wrapped first with white butcher paper and then with a spiral of 1.9 cm wide crimped curling ribbon spaced 1 cm apart without overlap. Purple curling ribbon was used because emerald ash borer females are attracted to that color (Francese et al. 2010) and it improved oviposition rate over other options tested in earlier studies (MA Keena, unpublished data). Beetles were held at 25 ± 2° C, 16:8 L:D and 65 ± 5% RH. Foliage was changed twice a week and eggs harvested once a week. Harvested eggs were held in Petri dishes (Falcon™ 100 × 15 mm) on a platform over water in a plastic box with a clear lid (30 cm × 70 cm × 20 cm) to maintain high humidity, and held at the same conditions as the adults until use or hatch. Eggs were checked daily during the week for hatch.

Diet infestation. When eggs were used to infest the diet, a piece of paper with 1–2 eggs (8–12 days old) was placed in the lens-shaped notch in the diet, with the edge of the paper wedged between the bottom of the dish and the diet, and with the egg facing toward the bottom and not in contact with the diet (Fig. 1). When neonate larvae (1-24 hours after hatch) were used to infest the diet, they were first checked under a dissecting microscope to ensure they appeared alive (not desiccated). A small slit in the diet was made near the notch, a few crumbs of diet were removed from the slit, and the larva was placed in the slit using a fine paint brush and carefully covered with a few crumbs of diet. When the lid was placed on the dish, care was taken to avoid exerting pressure on the larva. Working surfaces and tools were sterilized before use with a 2% benzalkonium chloride tincture (50% aqueous solution, mpbio.com, SKU 0215043191, diluted with 95% ethanol and water) or 5% solution of Krac Bac Plus (hillyard.com, Item 50); disposable gloves were worn to minimize diet contamination.

Petri dishes containing infested diet were stacked (8 dishes high) in 237 ml clear polystyrene jars, and the jar lids screwed on to hold the dishes in place. To orient the dishes vertically, the jars were laid on their sides with the diet notches oriented upward. The jars were placed in opaque tote boxes (45 × 28 × 23 cm) and held at the same environmental conditions as the adults.

Experiment 1. Diet 1 was the best diet tested in earlier trials with a slightly higher amount of methyl paraben (Diet N in Supplemental Table 2). In Diets 2 and 3, 40% and 100%, respectively, of the wheat germ in Diet 1 was

Figure 1. *Agrilus planipennis* diet in a Petri dish (view of the underside of the dish), showing the crescent-shaped notch and an egg on paper. Initial larval trails are visible.

replaced with soybean flour (50% protein, food grade). Soybean flour is readily available, comes with a standardized protein content (wheat germ is more variable, averaging around 25%)), and has been used in other beetle diets. In Diet 4, a higher level of sodium bicarbonate was added to the Diet 2 formula to reduce acidity, which necessitated a test of its effect on the function of the antimicrobial ingredients. The average moisture content of these diets was 48% by weight. Two diet layer treatments were also compared, a single 6 mm thick layer, and two 3 mm thick layers, with the second layer added after 5 weeks.

A minimum of 58 individuals was evaluated on each diet. Equal numbers of eggs or larvae from each family or group-mating cage were tested per diet to ensure the genetic variation was equally represented across families. Newly hatched larvae were used to infest 14% of dishes, while the remainder of the diet dishes was infested with eggs near hatch. After initial trials, egg infestation became the preferred method because it reduced the time to set up the experiment and avoided damage to larvae during transfer. A test was made to change the orientation of diet dishes during rearing from horizontal to vertical, with the egg placed at the top, based on the finding that emerald ash borer larvae primarily tunnel downward along the vertical plane of the trunk of healthy ash trees (Chen et al. 2011).

Hatch and larval survival were first checked after 21 days. The egg paper was removed, egg hatch was determined, and visual evidence of larval establishment was documented (frass and tunneling activity). Larval survival was assessed again visually at 5 weeks when the 6 mm diet was changed, if contaminated, and a second sheet of 3 mm diet was added to a 3 mm treatment. If mold or bacteria were found, the entire dish was discarded unless the larva was in a clean section of diet and could be transferred with low risk of contaminating a new diet dish. After 70 days larvae were excavated (Fig. 2), checked to determine if they had reached the J-shaped stage, indicating readiness to become pre-pupae, and if so were chilled to undergo simulated winter conditions. Morphological changes in larvae indicating they were ready for chill included a slight reduction in length and reduction to complete loss of intersegmental constrictions. At this stage larvae also became whiter, more opaque, and the thoracic segments were narrower and shorter than the abdominal segments. If the larva was not ready for chill at 70 days, it was reinserted into a furrow cut in its diet or in fresh diet (if the diet was extensively tunneled) and covered with diet crumbs or a flap of diet. In either case, the larva was placed in a space just large enough to accommodate it and covered with diet to prevent desiccation. Reinserted larvae were checked after 2–3 days to ensure they were still covered or had initiated tunneling again. They were checked weekly until ready for chill.

Larvae were chilled for 84 days at 10 ˚C in the tight fitting dishes with fresh diet. The chilling regime was chosen because it works well for *A. glabripennis* larvae reared on artificial diet (Keena 2005); an optimal chilling regime for emerald ash borer will be explored in future. Diet dishes were held horizontally during and after the chill period because the larvae were no longer actively tunneling. After chill, larvae were returned to rearing temperature (25 ± 2˚ C, 16:8 L:D and 65 ± 5 %RH) and allowed to resume feeding (reinserted into the diet as indicated above) or proceed to pupation. Once larvae clearly became pre-pupae (indicated by slight translucence behind the head and the head deeply retracted into the fleshy prothorax), they were moved to six-well plates (Falcon® 351146 Multiwell™), each well lined at the bottom with either filter paper or a 2 cm square piece of single-faced corrugated cardboard to provide a rough surface against which they could shed the larval and pupal skins. The plates were held over water in a box to maintain moisture. Pupae were weighed 24 hours after first being observed. Time to pupation was calculated as time from diet infestation to pupation, including the time in chill. After emergence, adults were held in the six-well plates until they could stand (about 4 days), then put in a 60 mm diameter x 15 mm deep Petri dish with an *F. uhdei* leaflet

Figure 2. An *Agrilus planipennis* larva and tunnel after 10 weeks in artificial diet.

until they began feeding. They were then segregated by sex, moved to mating containers, and allowed to feed 10 days before mating. The sex ratio of the adults was calculated for each diet.

For statistical comparison, survival data from individuals set up during each weekly interval (five weeks total) were grouped into separate cohorts. Because fewer than 10 individuals were set up per treatment during the final week of the experiment they were combined with the preceding week's cohort; there were 12-24 individuals per cohort, and four cohorts per treatment. Data from unhatched eggs were withdrawn from the calculations. Data were partially withdrawn (censored) if and when the diet became contaminated with microbes, the individual was killed due to human error, or mold mites [*Tyrophagus putrescentiae* (Schrank)] infested the dish. Proportions surviving from hatch to 21 and 70 days and surviving from hatch to pupation and eclosion as adults were calculated by dividing the number of individuals alive in the cohort by the number of individuals in that cohort still in the study (i.e., not withdrawn) at those points in time. The withdrawal category called "human errors" refers to any eggs or larvae damaged during handling or dying due to poor placement during infestation.

Experiment 2. In Experiment 2, the effect of diet moisture content on rearing success was evaluated using Diet 1, which was the best available diet at the time. The diet was presented as a 6 mm thick layer. The target moisture levels were 45%, 50%, and 55%, but the moisture ranges achieved were 46–47%, 49–52%, and 54–57%. Moisture content was determined with a moisture analyzer. A minimum of 87 individuals was evaluated at each moisture level. To reduce variability from potential genetic effects, equal numbers of individuals from each family or group-mated cage were used per diet. Eggs

near hatch were used to infest 98% of dishes, while the remainder was infested with neonates. Survival data, weights and sex ratios were determined as in Experiment 1 for each of the treatments. Four weekly cohorts each contained 14-37 individuals per treatment (individuals from the first two weeks were few and were therefore pooled).

Experiment 3. To evaluate larval growth and instar development on the best artificial diet (Diet 3), 875 larvae were individually reared for intervals of 1–10 weeks on the diet, then extracted, measured, and removed from the study (i.e., used for only one time interval). Between 80 and 105 individuals were reared per treatment interval. Neonates (n = 236) or eggs near hatch (n = 639) were used to infest diet, depending on availability. Once extracted from the diet, larval prothorax width, body length, and weight were recorded. Larvae to be measured at 3–10 weeks were provided rearing dishes filled to capacity with 6 mm thick diet, but to minimize effort to search for very small larvae, individuals to be measured only after 1–2 weeks were provided with diet filling only one quarter to one half of the rearing dish.

Larval weight was recorded when it exceeded 1.0 mg (lowest capacity of the balance used). Larvae were carefully held straight while the prothorax width and body length were measured to the closest 0.01 mm. Smaller larvae were measured using an ocular micrometer under a dissecting microscope and larger larvae were measured using an electronic digital caliper.

Instars were assigned based on body length and prothoracic width of wild larvae (Wang et al. 2005). The most accurate methods of instar assignment based on head capsule and urogomphus width were not used because these body parts cannot be properly measured, especially on smaller larvae, unless the larvae are killed or immobilized, and healthy larvae were required for use in other studies. Instar assignments are therefore estimates. Maximum and minimum limits for each instar were designated as the mean plus or minus one standard deviation for body length and two standard deviations for prothorax width, as considerable overlap exists in the prothoracic width ranges of each instar (Wang et al. 2005). If the two measurements conflicted and placed larvae in different instars, the more conservative prothorax parameter was used, but if body length exceeded the mean of the later instar, the later instar was assigned. If a larva died or could not be found, or the diet was contaminated with fungus or mites, the replicate was discarded.

Data analysis. To compare percentage survival (which was normally distributed) at each predetermined interval (21 and 70 d) and percentage that pupated or reached adulthood, the larvae were grouped into weekly cohorts (week placed on diet). The survival percentages for the four weekly cohorts were evaluated using ANOVA followed by Tukey HSD mean comparisons with $\alpha = 0.05$ (Statistix 2013). In Experiment 1, the model used diet formula, diet thickness and the interaction between the two as fixed effects, and in Experiment 2 diet moisture was the only fixed effect.

The fit of each data set to various distributions was evaluated using PROC UNIVARIATE (SAS Institute 2006) with the histogram option. The Shapiro-Wilk and the Anderson-Darling test were used to assess normality. However, in cases where no distributions met the normality assumption at $\alpha = 0.05$, we used the distribution that most closely emulated the data based on the tests for normality. The following dependent variables were analyzed in PROC GLIMMIX (SAS Institute 2006): time to pupation and pupal weight for insects in each diet in Experiments 1 and 2; and prothorax width, body length, and larval weight in Experiment 3. Time to pupation was the only discrete variable; the others were continuous.

In Experiment 1, a completely randomized design was used, with diet thickness, diet formula, sex, the interaction between diet thickness and formula, and the interaction between all three as fixed effects, and maternal family (either an individual female or a group cage) and diet moisture content as random

effects. In experiment 2, a completely randomized design was used, with diet moisture content, sex, and the interaction between the two as fixed effects, and maternal family (either an individual female or a group cage) as a random effect. The gamma distribution with a log link function was fitted to pupal weight data, while a negative binomial with a log link function was fitted to number of days to pupation. These distributions gave the best fit to these data, which had long right tails due to over dispersion.

In Experiment 3, a completely randomized design was used, with the week the larva was extracted (treatment), the assigned instar, and the interaction between the two as fixed effects and the diet batches and parental adult collection cohorts as random effects. A repeated measures analysis was not used because the larvae were destructively sampled, not returned to the diet after being measured, so each weekly cohort consisted of a different group of insects. The lognormal distribution with an identity link function was fitted to larval weights and a gamma distribution with a log link function was used for body length and prothorax measurements. The prothorax width measurements were multiplied by 10 before analysis to avoid taking the logs of decimal numbers. For each model, residuals were evaluated for normality and the homogeneity of variance. The group option was used in the random statement to account for unequal variances among groups (Experiment 2: sexes for pupal weights; Experiment 3: weeks for body length and instar prothorax width) if they existed. Differences among means were determined by the least-squares means test with $\alpha = 0.05$ and a Tukey-Kramer grouping (SAS Institute 2006).

Results

Experiment 1: substitution of soybean flour for wheat germ, and thickness of diet layer. Establishment (survival to 21d), survival to pupation, and survival to adult exceeded 81%, 35%, and 29%, respectively on all combinations of diet formulation and thicknesses (Table 2). There was no significant diet formulation or thickness effect on survival to 21 or 70 d, or survival to adult. There was a significant diet formulation effect and diet formulation by thickness interaction on percentage pupation: significantly more larvae pupated on 3 mm-thick Diet 3 than on 6 mm-thick Diet 3, on Diet 1 of either thickness, or on 3 mm-thick Diet 4.

Time to pupation and pupal weight did not differ significantly among the four diets or two diet thicknesses and there were no significant interactions between diet and thickness. Pupal weights, however, differed significantly by sex but no interaction was evident between diet thickness, diet formulation, and sex. Overall, female pupae weighed an average of 64.7 ± 2.5 mg and males weighed an average of 54.0 ± 1.7 mg.

Experiment 2: Diet moisture content. Establishment, survival to pupation, and survival to adult exceeded 77%, 24%, and 19%, respectively, across all tested diet moisture levels (Table 3). There was no significant effect of diet moisture content, sex, or the interaction between the two on survival to 21 or 70 d, or on percentage reaching pupation or adulthood.

There were significant effects of both diet moisture content and the interaction between it and sex on time to pupation, where the driest diet prolonged male development to pupation compared with diet containing 51% moisture. Males in the driest diet also developed significantly more slowly than females in that diet. Pupal weight was significantly affected by sex, with females weighing more than males, but it was not affected by diet moisture and there was no interaction between diet moisture and sex. Although moisture content had a significant effect only on days to pupation, the diet with 51% moisture was consistently better biologically than the others in terms of percentage survival to 21 and 70 d, pupation, and reared adults, and had the highest development

Table 2. Results of Experiment 1: Effects of Diets 1 – 4 and diet layer thickness on survival and development of *Agrilus planipennis.*

Diet thickness (mm)	Diet formulation	*n*	% Survival at 21 d (mean ± SE) [a]	% Survival at 70 d (mean ± SE) [a]	% Pupation (mean ± SE) [a]	% Adults (mean ± SE) [a]	Time to pupation (d) (mean ± SE) [bd]	Pupal weight (mg) (mean ± SE) [b]	Sex ratio F:M	Number of individuals withdrawn from study and reason: Egg failed to hatch	Microbes on diet	Human error	Trapped in plastic	Mites
3[c]	**1**	65	92.5 ± 5.5a	51.5 ± 6.9a	37.5 ± 3.5b	30.3 ± 3.3a	M 161 ± 11a F 162 ± 20a	M 56.8 ± 4.8a F 68.1 ± 9.1a	1:3	8	2	12	3	1
	2	68	97.0 ± 2.9a	68.0 ± 5.5a	49.3 ± 5.1ab	43.3 ± 6.0a	M 151 ± 11a F 149 ± 10a	M 52.6 ± 4.5a F 71.2 ± 5.8a	1:1	13	0	11	0	0
	3	66	94.5 ± 3.3a	72.3 ± 11.2a	67.8 ± 8.5a	51.0 ± 5.0a	M 176 ± 11a F 145 ± 9a	M 48.8 ± 3.5a F 71.2 ± 5.2a	1:1	5	7	8	0	0
	4	63	87.5 ± 6.0a	60.0 ± 5.6a	35.0 ± 3.0b	36.3 ± 9.3a	M 152 ± 12a F 159 ± 13a	M 56.6 ± 4.9a F 70.2 ± 6.4a	1:1	6	6	12	0	0
6	**1**	66	88.0 ± 6.2a	56.5 ± 9.3a	35.4 ± 8.0b	28.8 ± 4.9a	M 157 ± 11a F 198 ± 15a	M 52.2 ± 4.1a F 60.5 ± 5.4a	1:1	3	4	10	1	0
	2	63	81.3 ± 4.3a	62.3 ± 4.2a	35.8 ± 6.5b	31.5 ± 5.0a	M 150 ± 11a F 151 ± 13a	M 53.6 ± 4.9a F 75.7 ± 8.1a	1:2	6	2	10	0	0
	3	58	88.8 ± 5.7a	63.4 ± 4.1a	39.0 ± 2.5b	29.3 ± 6.4a	M 160 ± 10a F 153 ± 16a	M 54.9 ± 4.8a F 77.2 ± 9.0a	1:3	4	2	3	0	3
	4	59	95.8 ± 2.5a	74.3 ± 5.5a	56.3 ± 5.3ab	48.5 ± 7.3a	M 166 ± 11a F 170 ± 14a	M 56.5 ± 4.3a F 64.5 ± 6.2a	1:2	3	16	1	0	0

Table 2. Continued.

Diet thickness (mm)	Diet formulation	*n*	% Survival at 21 d (mean ± SE) [a]	% Survival at 70 d (mean ± SE) [a]	% Pupation (mean ± SE) [a]	% Adults (mean ± SE)[a]	Time to pupation (d) (mean ± SE)[bd]	Pupal weight (mg) (mean ± SE)[b]	Sex ratio F:M	Number of individuals withdrawn from study and reason				
										Egg failed to hatch	Microbes on diet	Human error	Trapped in plastic	Mites
Thickness:			$F_{1, 24} = 1.70$; $P = 0.2048$	$F_{1, 24} = 0.01$; $P = 0.9300$	$F_{1, 24} = 3.75$; $P = 0.0646$	$F_{1, 24} = 1.68$; $P = 0.2067$	$F_{1, 73} = 1.07$; $P = 0.3047$	$F_{3, 69} = 0.27$; $P = 0.8497$						
Sex:							$F_{1, 73} = 0.05$; $P = 0.8266$	$F_{1, 69} = 45.85$; $P < 0.0001$						
Diet* Thickness:			$F_{3, 24} = 2.09$; $P = 0.1281$	$F_{3, 24} = 1.37$; $P = 0.2762$	$F_{3, 24} = 4.75$; $P = 0.0097$	$F_{3, 24} = 2.75$; $P = 0.0648$	$F_{3, 73} = 0.48$; $P = 0.6976$	$F_{3, 69} = 0.68$; $P = 0.5669$						
Diet* Thickness* Sex:							$F_{7, 73} = 1.64$; $P = 0.1386$	$F_{7, 69} = 1.03$; $P = 0.4173$						

[a] Values followed in the same column by the same letter are not significantly different when analyzed by two-way analysis of variance followed by pairwise Tukey *HSD* comparisons with $\alpha = 0.05$ (Statistix 2013).
[b] Means in the same column followed by the same letter are not significantly different when analyzed by PROC GIMMIX (SAS Institute 2006) followed by Tukey-Kramer Least Squares Mean test with $\alpha = 0.05$. M = male and F = female
[c] Dishes initially contained a single 3 mm thick layer of diet; a second layer was added at 5 weeks.
[d] This time period includes the time in chill; the end point is variable as they did not begin chill or pupate simultaneously.

Table 3
Experiment 2: Effect of diet moisture on survival and development of *Agrilus planipennis* reared on Diet 1.

Diet moisture content (range)	*n*	% Survival at 21 d (mean ± SE)[a]	% Survival at 70 d (mean + SE)[a]	% Pupation (mean + SE)[a]	% Adults (mean ± SE)[a]	Time to Pupation (d) (mean ± SE)[bc]	Pupal weight (mg) (mean ± SE)[b]	Sex ratio F:M	Number of individuals withdrawn from study and reason				
									Egg failed to hatch	Microbes on diet	Human error	Trapped in plastic	Mites
47% (47)	100	77.7 ± 8.7a	44.8 ± 15.6a	31.9 ± 11.1a	20.5 ± 7.2a	M 193 ± 13a F 151 ± 5b	M 40.2 ± 5.5c F 72.2 ± 3.7a	5:1	19	2	7	8	3
51% (49-53)	87	90.5 ± 2.7a	58.0 ± 13.4a	41.3 ± 10.1a	29.0 ± 11.3a	M 142 ± 8b F 146 ± 4b	M 57.7 ± 4.6abc F 72.4 ± 4.0a	4:1	20	7	9	1	0
56% (54-57)	104	85.5 ± 6.6a	46.0 ± 12.2a	24.7 ± 11.1a	19.2 ± 8.6a	M 152 ± 9ab F 150 ± 15ab	M 51.6 ± 4.0bc F 81.7 ± 12.0ab	1:3	16	24	14	1	1
Diet:		$F_{2,9} = 0.96$; $P = 0.4201$	$F_{2,9} = 0.29$; $P = 0.7583$	$F_{2,9} = 0.58$; $P = 0.5773$	$F_{2,9} = 0.33$; $P = 0.7285$	$F_{2,13} = 5.92$; $P = 0.0149$	$F_{2,13} = 2.37$; $P = 0.1326$						
Sex:						$F_{1,13} = 2.34$; $P = 0.1504$	$F_{1,13} = 25.67$; $P = 0.0007$						
Diet*Sex:						$F_{2,13} = 3.96$; $P = 0.0454$	$F_{2,13} = 2.48$; $P = 0.1228$						

[a] Values followed in the same column followed by the same letter are not significantly different when analyzed by two-way analysis of variance followed by pairwise Tukey HSD comparisons with $\alpha = 0.05$ (Statistix 2013).
[b] Means in the same column followed by the same letter are not significantly different when analyzed by PROC GIMMIX (SAS Institute 2006) followed by Tukey-Kramer Least Squares Mean test with $\alpha = 0.05$. M = male and F = female
[c] This time period includes the time in chill and the end point is variable as they did not go into chill or pupate simultaneously.

rate to pupation. Microbial growth seemed most frequent on the diet with the highest moisture content, while more larvae were observed to die by tunneling into the rearing dishes when the diet was the driest. Adult sex ratio was skewed toward females on the two drier diets and toward males in the diet with the highest moisture content.

Experiment 3: phenology on best diet (Diet 3). Across all treatments, 77.9% of eggs hatched and 77.5% of larvae were alive when removed for examination. A total of 122 individuals were withdrawn (3.2% due to microbes, 9.0% due to human error, and 1.7% tunneled in plastic) and 167 larvae were dead when sampled. Significant interaction existed between weeks elapsed on diet and prothorax width, larval body length, and larval weight. Within an instar, larvae examined at earlier weeks tended to be smaller and weighed less than those examined in later weeks, as expected (Table 4). Average prothroax widths overlapped between instars two and three and between three and four except during weeks 7-10. There was considerable variation in the rate of development among larvae, but most reached the fourth instar by week 8 (Table 4). Larval sizes (lengths shown in Fig. 3) and weights overlapped between instars and the distributions of both in the fourth instar suggested a bimodal distribution, most likely due to differences between the sexes.

Discussion

The diet with the highest percentage survival to adult was Diet 3 at the 3mm initial thickness (Table 2). This diet also appeared to have reduced microbial problems based on lower numbers of larvae withdrawn from the study. The results show that the diet is sufficient to support full larval development without host material and can result in a high rate of successful pupation (67.8% pupation). This pupation rate is comparable to that achieved when larvae are reared in green ash bolts (68%) for parasitoid production but less than when tropical ash bolts are used in the process (Duan et al. 2013b). Current methods for holding pupae are not adequate and improvements to the larval chilling methods are needed to improve survival from egg hatch to adult. Several modifications in the diet and larval holding methods were needed to reach this level of survival and development: reducing antimicrobial components in the diet to the lowest functional level; changing protein sources in the diet from wheat germ, brewer's yeast, and casein to soy flour and casein; and reducing diet moisture content from 60% (when freshly made) to about 50%. Changing orientation of the dishes from horizontal to vertical and avoiding disturbance of the larvae (no diet change) until they reached the desired size likely also contributed to improved survival compared with diets tested before those presented here (see supplemental tables). Addition of a second layer of fresh diet at 5 weeks appeared to improve success compared with provision of a thicker layer of diet for the duration of the larval stage. Adults grown on Diet 3 mate and lay viable eggs, and at the time of submission of this paper, the sixth continuous generation of larvae is completing development on it. Further improvement in the diet may be possible, and improved pupal holding methods are needed to prevent death of prepupae and pupae possibly tied to incomplete ecdysis.

Development of emerald ash borer larvae in *Fraxinus* spp. both in the United States and China varies from univoltine to two-year life cycles. This variation was tied to latitude in China, where larvae require two years in the north and only one year in the south to complete development (Zhao et al. 2005, Wie et al. 2007). In the United States, host condition was shown to influence the beetle's development rate; interruption of phloem transport due to girdling or high gallery densities resulted in faster larval development (Tluczek et al. 2011). Total duration of the larval stage averaged 300 days in ash in China, and time to reach the fourth instar averaged 40 days (Wang et al. 2005). When larvae were reared in tropical ash bolts held at 25 °C for production of parasitoids, 50% of the larvae

Table 4
Experiment 3: Size and weight parameters (mean ± SE) by instar of *Agrilus planipennis* larvae reared for various numbers of weeks in Diet 3.

	Prothorax Width (mm) by Instar [a]				**Body Length (mm) by Instar [a]**				**Larval Weight (mg) by Instar [a]**		
Week	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**2**	**3**	**4**
1	0.44 ± 0.01g (40)[b]	0.65 ± 0.03f (15)			3.1 ± 0.1e (40)	3.9 ± 0.3de (15)					
2	0.41 ± 0.02g (11)	0.70 ± 0.03f (27)	1.30 cde (1)		3.4 ± 0.2de (11)	5.5 ± 0.3d (27)	7.7 dc (1)				
3	0.40 ± 0.04g (2)	0.88 ± 0.04ef (37)	1.33 ± 0.08cd (7)	2.33 abc (1)	2.4 ± 0.4e (2)	6.2 ± 0.2d (37)	11.0 ± 0.9c (7)	17.4 abc (1)			
4	0.54 fg (1)	0.97 ± 0.05ef (22)	1.52 ± 0.05cd (23)	2.36 ± 0.28abc (2)	3.4 de (1)	7.0 ± 0.5d (22)	11.9 ± 0.8c (23)	15.2 ± 2.7abc (2)	2.2 ± 1.2cd (8)	6.7 ± 1.2bc (12)	22.2 ± 1.7abc (1)
5		1.01 ± 0.06def (9)	1.53 ± 0.05cd (27)	2.35 ± 0.14abc (9)		7.2 ± 0.6cd (9)	12.0 ± 0.8c (27)	20.7 ± 1.9abc (9)	3.7 ± 1.3cd (5)	7.2 ± 1.1bc (24)	29.7 ± 1.3ab (6)
6		0.98 ± 0.12def (2)	1.80 ± 0.07c (18)	2.32 ± 0.11bc (21)		8.8 ± 1.4cd (2)	15.4 ± 0.8abc (18)	22.9 ± 1.1a (21)	6.2 bcd (1)	16.6 ± 1.8bc (17)	37.9 ± 1.1ab (21)
7		0.86 ± 0.08ef (3)	1.66 ± 0.07c (14)	2.77 ± 0.12abc (30)		5.0 ± 0.7de (3)	15.1 ± 1.2bc (14)	25.2 ± 1.5a (30)	1.5 cd (1)	11.8 ± 1.2bc (14)	50.0 ± 1.1ab (27)
8			1.75 ± 0.14c (4)	2.81 ± 0.11ab (42)			12.5 ± 1.5bc (4)	27.3 ± 1.4a (42)		13.2 ± 1.3bc (4)	70.7 ± 1.1a (42)
9		0.55 fg (1)	1.68 ± 0.10c (7)	3.11 ± 0.13a (34)		4.3 de (1)	14.4 ± 1.3bc (7)	29.2 ± 1.5a (34)		10.0 ± 1.3bc (6)	98.3 ± 1.1a (34)
10			2.03 ± 0.19bc (3)	3.02 ± 0.12a (38)			16.6 ± 2.2abc (3)	27.8 ± 1.0a (38)		15.5 ± 1.5bc (2)	87.8 ± 1.1a (38)
Week:		$F_{9, 385} = 4.26$; $P < 0.0001$					$F_{9, 38} = 4.59$; $P = 0.0004$			$F_{8, 227} = 4.13$; $P = 0.0001$	
Instar:		$F_{3, 16} = 135.71$; $P < 0.0001$					$F_{3, 333} = 111.22$; $P < 0.0001$			$F_{2, 10} = 70.58$; $P < 0.0001$	
Week* Instar:		$F_{16, 385} = 4.20$; $P < 0.0001$					$F_{16, 333} = 3.75$; $P < 0.0001$			$F_{10, 227} = 3.55$; $P = 0.0002$	

Means followed by different letters within a parameter were significantly different when analyzed by PROC GIMMIX (SAS Institute 2006) followed by Tukey-Kramer Least Squares Mean test with α = 0.05. Larvae were weighed only if they were undamaged and exceeded 1 mg.
[a] Instars were assigned according to Wang et al. (2005).
[b] Numbers in parentheses indicate number of larvae.

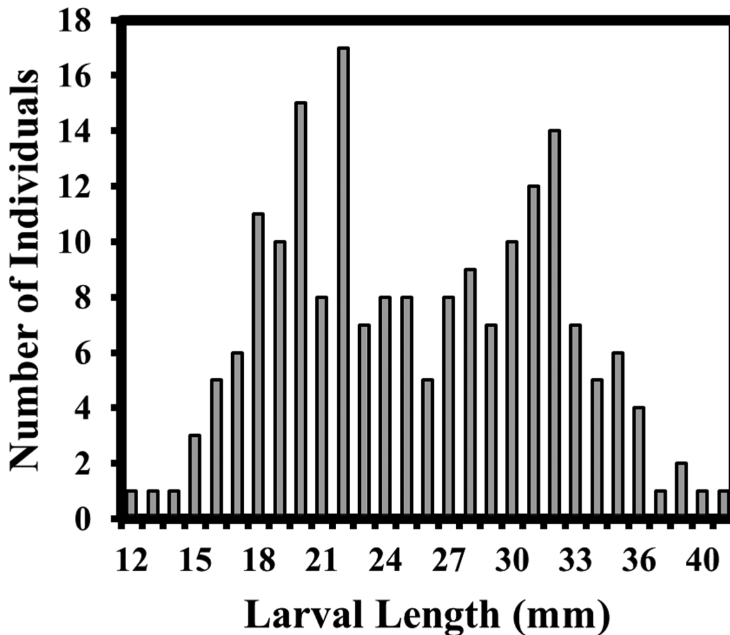

Figure 3. Experiment 3: Distribution of *Agrilus planipennis* fourth instar larval lengths reared in Diet 3.

were in the fourth instar by day 39 (Duan et al. 2013a). On Diet 3 at 25 °C, >70% of the larvae were in the fourth instar by week 6 (42 days) and the average time to pupation was 175 days (which included 84 days of chill at 10° C). Thus total larval development time on the artificial diet is significantly reduced over that in live ash trees even though time to the fourth instar is comparable to that in ash phloem (either living trees or cut bolts). In addition, larval dimensions for each instar reared on Diet 3 are comparable to those obtained for larvae grown in ash phloem, and the bimodal length distribution in the fourth instar, shown to result from differences between the sexes (Cappaert et al. 2005, Wang et al. 2005), is also present in diet-reared larvae.

Antimicrobial compounds are often incorporated into artificial diets for insects despite detrimental effects on many insect species, such as slowed larval development, increased mortality (particularly of embryos, which are the most sensitive stage), and wing deformation (Singh and Bucher 1971, Dunkel and Read 1991, Cohen 2004). Thus, a concentration of antimicrobials sufficient to control microbial contaminants while minimizing adverse effects on the insect is a delicate balance, complicated by variable sensitivity to antimicrobials among species (Cohen 2004). The effectiveness of antimicrobials used in the emerald ash borer diets is probably also strongly affected by the pH of the diet, as antimicrobials are more effective at lower pH and almost completely ineffective at pH 7.0 and above (Cohen 2004). The reported effective levels of methyl paraben, sorbic acid, and sodium propionate are 1000, 800, and 800 ppm, respectively (Cohen 2004). The levels of these antimicrobials in emerald ash borer Diet 3 (600 ppm for each) are all lower than the effective rate, so extra care was taken to minimize microbial contamination. Emerald ash borer appears to be very sensitive to the antimicrobials in the diet, possibly due to negative impacts they may have on their identified

digestive symbionts (Vasanthakumor et al. 2008). In addition, when the pH of the diet was artificially increased with the addition of more sodium bicarbonate (Diet 4) a marked (although not significant) improvement occurred in survival on the 6 mm diet, but loss due to fungal contamination also increased dramatically, although it often did not kill the larva. This suggests further reduction of antimicrobial compounds or replacement with compounds better tolerated by the larvae or their symbionts could improve survival on the emerald ash borer diet.

Comparison of the composition of an earlier diet formulation to ash phloem indicated the diet formulation had significantly more protein than the phloem. Chen et al. (2011) found reducing protein by either eliminating yeast or casein from emerald ash borer diet reduced mortality. In addition, eliminating yeast was found to increase biomass (Chen et al. 2011), while other insects became stressed if the protein content of their diet was too high (Cohen 2004). The protein sources in the initial emerald ash borer diet were wheat germ, casein, and brewer's yeast. Earlier experiments (supplemental tables) showed eliminating only one of the protein components was not sufficient to improve larval survival and that wheat germ either acts as a source of diet contamination or encourages fungal growth, suggesting its elimination could reduce loss due to microbes. Experiment 1 showed that a diet without yeast where the wheat germ is replaced with soy flour (and some potato starch to compensate for the loss of carbohydrates), which is 50% protein but higher in potassium, vitamin A, fats, and essential amino acids (Cohen 2004), improved larval survival and development over the previous diets (supplemental tables). These reductions in number and changes in protein sources (the only sources of iron in the diet) also lowered iron content of the diet, which was initially higher than in green ash phloem, and may have helped improve larval performance. Higher iron content in the diet of Asian longhorned beetle, *A. glabripennis*, was shown to slow larval development and reduce pupation (Keena 2005). Emerald ash borer may also be sensitive to iron levels in the diet but further testing is necessary to confirm this.

In previous studies by Chen et al. (2011), the amount of water incorporated into emerald ash borer diet (moisture content of prepared diet was not measured) was shown to affect larval weight and survival. Excess or insufficient water in emerald ash borer diet increased mortality, and larval biomass increased with water content (Chen et al. 2011). Larvae of *Capnodis*, another buprestid, require higher moisture content in the diet when first hatched (60–62% moisture) than later instars (Gindin et al. 2009). Water content of phloem of different ash species ranges from 44 to 52% (Hill et al. 2012) while water content of emerald ash borer diet reported here was about 60% before drying. Emerald ash borer neonates placed on diet containing 60% moisture died quickly, possibly from drowning. A gradient of diet moisture was likely created when the diet was dried, with the least moisture in the outer surfaces and the most in the center. This allowed larvae to choose the moisture level most favorable to them. Most larvae tunneled into the diet, which appeared to reduce the risk of mortality of small larvae from being trapped in air pockets outside the diet. In Experiment 2, although the only significant difference in diets varying in moisture was time to pupation, larvae grown on diet containing more moisture tended to be heavier, while the most survived to adult on the diet with intermediate (51%) moisture. We conclude that diets with moisture content close to 50% provide the best environment for emerald ash borer larvae and hold their moisture long enough in the dishes for larvae to complete development. Water retention in the diet is important because it obviates the need to replace diet during the larval stage; in cases where diet needed to be changed, more than 30% of the larvae had difficulty reestablishing.

Orientation of diet dishes and stage at infestation were also critical to survival and development of the larvae. Because emerald ash borer larvae are adapted to move along a vertical plane under ash bark, primarily tunneling downward in healthy trees (Chen et al. 2011), orienting the dishes vertically instead of horizontally, as shown in prior experiments, allowed the larvae to move

and feed along a natural plane. In horizontal Petri dishes the larvae, especially the younger instars, often tunneled downward and died after initiating a tunnel into the plastic bottom. Larger larvae appeared to more readily discern the surface of the Petri dish and turned to avoid it. When dishes were held in the vertical position, larval and egg infestation techniques were equally successful. Egg infestation was less time-consuming but more prone to problems. If the paper substrate holding the egg wicked moisture from the diet or was dislodged and brought the egg into contact with the diet, the embryo often failed to hatch, possibly due to drowning or adverse effects of the antimicrobials in the diet. Emerald ash borer eggs are resistant to desiccation (Rigsby et al. 2013), which could make them less able to eliminate excess absorbed moisture from the diet. Also, if the paper became lodged between the dish and the diet, the egg was often damaged or the larva dropped into an air pocket and died. The legless larvae appeared to lose traction when outside a solid substrate. Larval infestation presented its own problems. Larvae were crushed if diet crumbs placed over them were too compacted when the lid was put on. Larvae were less likely to tunnel into the plastic and die when placed in the diet with the head facing the side of the dish rather than the bottom or top. The notch in the diet became a repository for frass and was often used by larger larvae as a molting chamber. Similar chambers may have improved rearing of larger *Capnodis* larvae, which require rearing on crumbled rather than solid diet (Gindin et al. 2009).

An artificial diet and individual rearing system was developed that can be used as a basis in studies requiring standardized, replicable rearing media without the added effort and variability imposed by the need for host material. The best-performing emerald ash borer diet from this study is being used to test effects of ash allelochemicals on emerald ash borer larvae in ongoing studies aiming to understand mechanisms of ash resistance to emerald ash borer (Cipollini et al. 2011, Whitehill et al. 2014). In the near future, the possibility of using the diet to evaluate resistant ash breeding stock developed by Koch et al. (2012) will be tested by incorporating bark/phloem from the trees into the diet and monitoring emerald ash borer larval survival. A rearing system incorporating this diet can provide larvae of relatively uniform age and size not only for mass production but also for pesticide and other bioassays. The standard diet and rearing system will also facilitate studies to determine the effects of temperature on larval development and development of a phenology model to predict when each stage will be present and how fast populations will increase. Currently, a laboratory strain is being developed to provide a predictable year round supply of emerald ash borer while the rearing methods are being improved. Lastly, the artificial diet presented here provides some hope for the development of a mass production system for the emerald ash borer and its biological control agents.

Acknowledgments

We thank D. Agresti, J. Biroscak, P. Moore, V. Sánchez, B. Secskas, and A. Vandel for rearing assistance at the Forest Service quarantine facility in Ansonia, CT. We thank L. Phelan, S. Opiyo, and T. Burgos-Hernandez at the Ohio State University for assisting with a nutritional analysis of an earlier diet so we could compare it with a published ash phloem analysis. We thank I. Fraser and her crew at the Animal and Plant Health Inspection Service location in Brighton, MI, and L. Bauer and D. Miller at the Forest Service in East Lansing, MI, for obtaining and shipping the adults used as sources of eggs. We thank J. Stanovick for statistical advice. We thank R. Bullock, J. Dedes, J. Hansen, V. Sánchez and the anonymous reviewers for offering suggestions that improved this manuscript. This work was funded in part by Animal and Plant Health Inspection Service through two interagency agreements (07-IA-11242300-151 and 10-IA-11242303-075).

Literature Cited

Aukema, J. E., B. Leung, K. Kovacs, C. Chivers, K. O. Britton, J. Englin, S. J. Frankel, R. G. Haight, T. P. Holmes, A. M. Liebhold, D. G. McCullough, and B. V. Holle. 2011. Economic impacts of non-native forest insects in the continental United States. PloS One 6: e24587 doi:10.1371/journal.pone.0024587.

Bauer, L. S., H. Liu, D. Miller, and J. Gould. 2008. Developing a classical biological control program for *Agrilus planipennis* (Coleoptera: Buprestidae), an invasive ash pest in North America. Newsletter of the Michigan Entomological Society 53: 38–39.

Blossey, B., D. Eberts, E. Morrison, and T. R. Hunt. 2000. Mass rearing the weevil *Hylobius transversovittatus* (Coleoptera: Curculionidae), biological control agent of *Lythrum salicaria*, on semiartificial diet. Journal of Economic Entomology. 93: 1644–1656.

Cappaert, D., D. G. McCullough, T. M. Poland, and N. Siegert. 2005. Emerald ash borer in North America: a research and regulatory challenge. American Entomologist 51: 152–165.

Carey, J. R. 2001. Insect biodemography. Annual Review of Entomology 46: 79–110.

Chen, Y., T. Ciaramitaro, and T. Poland. 2011. Moisture content and nutrition as selection forces for emerald ash borer larval feeding behavior. Ecological Entomology 36: 344–354.

Cipollini, D., Q. Wang, J. G. A. Whitehill, J. Powell, P. Bonello, and D. A. Herms. 2011. Distinguishing defensive characteristics in the phloem of ash species resistant and susceptible to emerald ash borer. Journal of Chemical Ecolology 37: 450–459.

Cohen AC. 2004. Insect diets science and technology. CRC Press, Boca Raton, FL.

Duan, J. J. and C. Oppel. 2012. Critical Rearing Parameters of Tetrastichus planipennisi (Hymenoptera: Eulophidae) as Affected by Host Plant Substrate and Host-Parasitoid Group Structure Journal of Economic Entomology 105: 792-801.

Duan, J. J., K. Larson, T. Watt, J. Gould, and J. P. Lelito. 2013a. Effects of Host Plant and Larval Density on Intraspecific Competition in Larvae of the Emerald Ash Borer (Coleoptera: Buprestidae). Environmental Entomology 42: 1193-1200.

Duan, J. J., T. Watt, K. Larson, P. Taylor, and J. P. Lelito. 2013b. Effects of ambient temperature on egg and larval development of the invasive emerald ash borer (Coleoptera: Buprestidae): implications for laboratory-rearing. Journal of Economic Entomology 106: 2101-2108.

Dunkel, F. V., and N. R. Read. 1991. Review of the effect of sorbic acid on insect survival in rearing diets with reference to other antimicrobials. American Entomologist 37: 172–178.

Francese, J. A., D. Crook, I. Fraser, D. R. Lance, A. J. Sawyer, and V. C. Mastro. 2010. Optimization of trap color for emerald ash borer (Coleoptera: Buprestidae). Journal of Economic Entomology 103: 1235–1241.

Gindin, G., T. Kuznetsova, A. Protasov, S. Ben Yehuda, and Z. Mendel. 2009. Artificial diet for two flat-headed borers, *Capnodis* spp. (Coleoptera: Buprestidae). European Journal of Entomology 106: 573–581.

Gould, J., J. Tanner, D. Winograd, and S. Lane. 2004. Initial studies on the laboratory rearing of emerald ash borer and foreign exploration for natural enemies. Proceedings Emerald Ash Borer Research and Technology Development Meeting, Romulus, Michigan, Oct. 5–6, 2004. USDA Forest Service Publication FHTET-2004-15.

Haack, R. A., E. Jendek, H. P. Liu, R. K. Marchant, T. Petrice, M. T. Poland, and H. Ye. 2002. The emerald ash borer: A new exotic pest in North America. Newsletter of the Michigan Entomological Society 47: 1–5.

Herms, D. A., D. G. McCullough, D. R. Smitley, C. Sadof, R. C. Williamson, and P. L. Nixon. 2009. Insecticide options for protecting ash trees from emerald ash borer. North Central IPM Center Bulletin. 12 pp.Available online: http://www.emeraldashborer.info/files/Multistate_EAB_Insecticide_Fact_Sheet.pdf

Herms, D. A. and D. G. McCullough. 2014. Emerald ash borer invasion of North America: History, biology, ecology, impacts, and management. Annual Review of Entomology 59: 13-30.

Hill, A. L., J. G. A. Whitehill, S. O. Opiyo, P. L. Phelan, and P. Bonello. 2012. Nutritional attributes of ash (*Fraxinus* spp.) outer bark and phloem and their relationships to resistance against the emerald ash borer. Tree Physiology 32: 1522–1532.

Keena, M. A. 2005. Pourable artificial diet for rearing *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and methods to optimize larval survival and synchronize development. Annals of the Entomological Society of America 98: 536–547.

Koch, L. K., D. W. Carey, K. S. Knight, T. Poland, D. A. Herms, and M. E. Mason. 2012. Breeding strategies for the development of emerald ash borer – resistant North American ash. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees General Technical Report *PSW-GTR-240*. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture pages 235–239. Available from http://www.fs.fed.us/psw/publications/documents/psw_gtr240/psw_gtr240_235.pdf (accessed December 2014).

McCullough, D. G., T. M. Poland, A. C. Anulewicz, and D. Cappaert. 2009. Emerald ash borer (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) attraction to stressed or baited ash trees. Environmental Entomology 38: 1668–1679.

Mourikis, P. A. and P. Vasilaina-Alexopoulou. 1975. Über die Laborzucht und Entwicklung des Pfirsich-Prachtkaefers, *Capnodis tenebrionis* L. (Col., Buprestidae). Anzeiger für Schädlingskunde Pflanzenschutz Umweltschutz 48: 75–77.

Poland, T. M. and D. G. McCullough. 2006. Emerald ash borer: invasion of the urban forest and the threat to North America's ash resource. Journal of Forestry 104: 118–124.

Rebek, E. J., D. A. Herms, and D. R. Smitley. 2008. Interspecific variation in resistance to emerald ash borer (Coleoptera: Buprestidae) among North American and Asian ash (*Fraxinus* spp.). Environmental Entomology 37: 242–246.

Rigsby, C. M., D. Cipollini, E. M. Amstutz, T. J. Smith, and J. A. Yoder. 2013. Water conservation features of ova of *Agrilus planipennis* (Coleoptera: Bupresidae). Environmental Entomology 42(2): 363–369.

SAS Institute, Inc. 2006. SAS OnlineDoc®. Version 9.1.3. Available from http://support.sas.com/onlinedoc/913/docMainpage.jsp (accessed December 2014).

Singh, P., and G. E. Bucher. 1971. Efficacy of 'safe' levels of antimicrobial food additives to control microbial contaminants in a synthetic diet for *Agria affins* larvae. Entomologia Experimentalis et Applicata 14: 297–309

Statistix. 2013. Statistix for windows user's manual. Analytical Software, Tallahassee, FL.

Tluczek, A. R., D. G. McCullough, and T. M. Poland. 2011. Influence of host stress on emerald ash borer (Coleoptera: Buprestidae) adult density, development, and distribution in *Fraxinus pennsylvanica* trees. Environmental Entomology 40: 357–366.

(USDA) U.S. Department of Agriculture. 2013. Emerald ash borer biological control release and recovery guidelines. Available from http://www.nrs.fs.fed.us/disturbance/invasive_species/eab/local-resources/downloads/EAB-Biocontrol-Field-Guidelines-2013.pdf. (accessed 5 December 2014).

(USDA-APHIS) U.S. Department of Agriculture, Animal Plant Health Inspection Service. 2014. EAB states federal quarantine is in effect. Available from http://www.hungrypests.com/the-spread/ByPest.php?pestabbr=eab (accessed 5 December 2014).

Vasanthakumar, A., J. Handelsman, P. D. Schloss, L. S. Bauer, and K. F. Raffa. 2008. Gut microbiota of an invasive subcortical beetle, *Agrilus planipennis* Fairmaire, across various life stages. Environmental Entomology 37: 1344-1353.

Wang, X.Y., Z. Q. Yang, G. J. Liu, and E. S. Liu. 2005. Larval instars and stadia of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). Scientia silvae Sinicae 41: 97–102.

Wei, X., Y. Wu, R. D. Reardon, T. H. Sun, M. Lu, and J. H. Sun. 2007. Biology and damage traits of emerald ash borer (*Agrilus planipennis* Fairmaire) in China. Insect Science 14: 367–373.

Whitehill J. G. A., A. Popova-Butler, K. B. Green-Church, J. L. Koch, D. A. Herms, and P. Bonello. 2011. Interspecific proteomic comparisons reveal ash phloem genes potentially involved in constitutive resistance to the emerald ash borer. PLoS ONE 6(9): e24863. doi:10.1371/journal.pone.0024863.

Whitehill, J. G. A., S. Opiyo, D. Cipollini, J. L. Koch, D.A. Herms, and P. Bonello. 2012. Interspecific comparison of constitutive ash phloem phenolic chemistry reveals compounds unique to Manchurian ash, a species resistant to emerald ash borer. Journal of Chemical Ecology 38: 499-511.

Whitehill, J. G. A., C. Rigsby, D. Cipollini, D. A. Herms, and P. Bonello. 2014. Decreased emergence of emerald ash borer from ash treated with methyl jasmonate is associated with induction of general defense traits and the toxic phenolic compound verbascoside. Oecologia 176: 1047–1059.

Zhao, T. H., R. T. Gao, H. P. Liu, L. S. Bauer, and L. Q. Sun. 2005. Host range of emerald ash borer, *Agrilus planipennis* Fairmaire, its damage and the countermeasures. Acta Entomologia Sinica 48: 594–599

Supplemental Table 1. *Agrilus planipennis* diets tested before those detailed in this paper. Pre-mixed groups of ingredients (in grams) are presented in sequence of addition during diet preparation (see text for details).

	Ingredient	**Diet Trial 1**			
	(vendor, item #)	**A***	**B**	**C**	**D**
Starting Base	Distilled water	5,000	5,000	5,000	5,000
	Agar *Gracilaria* species (mooragar.com, 41005)	220	220	220	220
	Sodium bicarbonate (household baking soda)	5	5	5	5
Dry Mix #1	Casein (Americancasein.com, AC-130)	190	95	48	
	Sucrose (table sugar)	315	315	315	315
	Wesson salt mix without Fe (testdiet.com, 25671)	56	56	56	56
	Sorbic acid (insectrearing.com, 6967)	10	10	10	10
	Calcium propionate (sigmaaldrich.com, 18104)				
	Sodium propionate (sigmaaldrich.com, P1880)	10	10	10	10
	Methyl paraben (insectrearing.com, 7685)	5	5	5	5
	Potato starch (nuts.com)				
Dry Mix #2	Wheat germ (insectrearing.com, 1659)	630	473	315	158
	Soybean flour (insectrearing.com, 1500)				
	Brewer's yeast (insectrearing.com, 1700)	225	180	135	90
Wet Mix	Cholesterol (insectrearing.com, 5180)	19	19	19	19
	Wheat germ oil (insectrearing.com, G5960)	24	30	36	42
Vitamins and Fiber	Choline chloride (insectrearing.com, 6105)	4	4	4	4
	Vitamin A acetate beadlets (ZMC-USA.com, 325 CWS/GFB)	5	5	5	5
	Vitamin mix (insectrearing.com, F8128)	71	71	71	71
	Cellulose (insectrearing.com, 3425)	1,625	1,916	2,160	2,404

Vendor website and product number are provided in parentheses after each ingredient.
*Best diets available in each trial against which others were compared.

Supplemental Table 1. Continued.

Diet Trial 2				Diet Trial 3				Diet Trial 4			
E*	**F**	**G**	**H**	**I***	**J**	**K**	**L**	**M**	**N***	**O**	**P**
5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000
175	175	175	175	220	220	220	220	220	220	220	220
5	5	5	5	5	5	5	5	5	5	5	5
190	190	190	190	200	200	200		200	200	200	200
315	315	315	315	300	300	300	300	300	300	300	300
56	56	56	56	50	50	50	50	45	45	45	45
10	10		10	5	5	5	5	5	5	5	5
				5	5	5	5	5	5	5	5
10	10	10									
5		5	5	5	5	5	5	3	3	3	3
					500	450	200				
630	630	630	630	500		500	500	750	500	250	
225	225	225	225	450	450		450	450	450	450	450
19	19	19	19	19	19	19	19	19	19	19	19
24	24	24	24	24	24	24	24	24	24	24	24
4	4	4	4	4	4	4	4	4	4	4	4
5	5	5	5	5	5	5	5	5	5	5	5
71	71	71	71	71	71	71	71	71	71	71	71
1,625	1,625	1,625	1,625	1,500	1,500	1,500	1,500	1,500	1,500	1,500	1,500

Supplemental Table 2.
Survival and development of *Agrilus planipennis* on diet formulations in earlier diet trials (diet compositions are given in supplemental Table 1).

Diet Trial	Diet formulation	*n*	% Survival at 21 d	% Survival at 70 d	% Pupation	% Adults	Number of individuals withdrawn from study and reason					Diet Infest Stage	Orientation of diet dishes	Average % Moisture
							Egg failed to hatch	Microbes on diet	Human error	Trapped in plastic	Mites			
1	A	94	45.7	6.4	0.0	0.0	0	0	19	2	0	100% larvae	Horizontal	44
	B	91	44.0	2.0	0.0	0.0	0	0	21	2	0			
	C	92	50.3	0.0	0.0	0.0	0	0	22	0	0			
	D	92	48.3	2.0	0.0	0.0	0	0	19	0	0			
2	E	124	67.5	9.4	0.0	0.0	12	0	23	4	0	78% larvae and 12% eggs	Horizontal	51
	F	125	71.0	13.0	6.0	6.0	9	4	22	5	0			
	G	128	65.0	5.5	0.0	0.0	15	0	18	3	0			
	H	121	37.2	4.3	0.0	0.0	6	4	6	11	0			

Supplemental Table 2. Continued.

Diet Trial	Diet formulation	*n*	% Survival at 21 d	% Survival at 70 d	% Pupation	% Adults	Number of individuals withdrawn from study and reason: Egg failed to hatch	Microbes on diet	Human error	Trapped in plastic	Mites	Diet Infest Stage	Orientation of diet dishes	Average % Moisture
3	**I**	117	89.3	25.8	17.2	17.2	29	19	19	11	0	36% larvae and 64% eggs	Vertical	40
	J	114	90.0	16.0	12.0	8.0	27	12	15	7	0			
	K	111	83.4	25.3	12.7	10.1	27	12	11	4	0			
	L	114	81.9	28.2	24.2	16.1	33	12	21	1	0			
4	**M**	64	80.2	56.6	42.4	14.1	27	13	2	3	0	2% larvae and 98% eggs	Vertical	44
	N	64	78.0	53.0	20.0	13.0	27	9	3	2	0			
	O	62	81.1	5.0	5.0	5.0	27	2	4	2	0			
	P	64	89.9	33.2	19.0	9.5	26	2	5	5	0			

Trial 1: Comparison of four levels of protein.
Trial 2: Comparison of different combinations of antimicrobial ingredients and 20% reduction in agar content.
Trial 3: Comparison of different combinations of protein sources; excluded protein sources were replaced with an equal weight of potato starch; sodium propionate was replaced with calcium propionate to reduce sodium content and harden the agar gel; the antimicrobials sorbic acid and calcium propionate were reduced in weight by 50%; and amounts of cellulose, casein, salt mix, and wheat germ were lowered if present.
Trial 4: Comparison of four levels of wheat germ and lower levels of methyl paraben and salt mix.
Low diet moisture contents in trials 1, 2, and 4 may have contributed to low survival.