

SPECIAL ISSUE: POPULATION STRUCTURE AND
DYNAMICS OF INVASIVE SPECIESAn artificial delay in emergence influences the number
but not the fitness of adult emerald ash borer emerging
from infested ash woodWilliam E. Fick & Chris J. K. MacQuarrie* Natural Resources Canada, Canadian Forest Service, Great Lakes Forestry Centre, 1219 Queen St. East, Sault Ste. Marie,
Ontario, Canada

Accepted: 19 November 2017

Key words: phenology, fat content, water content, body condition, rearing, protandry, propagule pressure, invasive species, Coleoptera, Buprestidae, *Agrilus planipennis***Abstract**

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is the most significant invasive forest pest in North America. Laboratory research on this species requires sources of adult and larval insects that are of the same fitness as those present in the wild. Production of adult EAB relies on flushing adults from logs which are subject to cold storage for some period prior to use. The effect of this storage on the number of insects emerging or the fitness of those that emerge has not been investigated. We subjected logs of EAB-infested white ash, *Fraxinus americana* L. (Oleaceae), to 7–14 months of cold storage and quantified the number of insects that emerged, the time to emergence, and the body condition of adults as a measure of fitness. Body condition was evaluated using Soxhlet fat extraction and water weight. No published methods for Soxhlet fat extraction were available for this species so we developed extraction protocols. The number of insects emerging decreased with time, but fitness (i.e., fat and water content) did not decrease. Time to emergence did increase but only in the longest storage treatment, whereas a comparison of male vs. female emergence provides evidence for protandry in EAB. Rearing programs for EAB using wood from cold storage should adjust the amount used to produce a given number of insects but the quality of those individuals emerging will not be affected. We suggest that those insects that perished during storage were of lower quality when entering diapause and thus would serve as poor-quality host in rearing programs for natural enemies of EAB. These data also provide evidence for one pathway of introduction for EAB, suggesting that refrigeration in transit was required for EAB to remain viable and establish its beachhead in North America.

Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a forest pest that has spread rapidly and has caused large-scale destruction within the naïve populations of ash (*Fraxinus* spp., Oleaceae) that occur in North America and European Russia (Hermes & McCullough, 2014; Orlova-Bienkowskaja, 2015). This exotic buprestid was first discovered in North America in 2002 in southeastern Michigan, USA, and Windsor, Ontario, Canada (Haack et al., 2002), though it was likely introduced sometime in the 1990s (Siegert et al.,

2014). The insect is now found throughout much of the eastern and midwestern USA, throughout southern Ontario, eastern Quebec, and as far north as Winnipeg, MB, Canada, and Quebec City, QC, Canada, and as far west as Colorado, USA (USDA, 2017).

Research in the laboratory on EAB has been limited by insect availability. Adult EAB can be collected from the field between May and July throughout most of the invaded range (Cappaert et al., 2005). Outside of that period, beetles are obtained by harvesting infested ash trees in the late fall or early winter after the cessation of larval development. Logs from these trees are placed in cold storage for some months and brought into a warm flushing room when beetles are required (e.g., Chen & Poland, 2009; Timms et al., 2006). More recently, techniques have

*Correspondence: E-mail: christian.macquarrie@canada.ca

been developed to rear EAB from eggs in small-diameter ash log 'mini-bolts' for the production of parasitoids (e.g., Ulyshen et al., 2010) or for the establishment of an ongoing laboratory colony where the insects are maintained through subsequent generations. When produced for EAB colonies these mini-bolts are often stored for some period after the larvae enter diapause, until adults are required for experiments. Although these methods are common in the study of EAB, the effect of long-term storage on EAB fitness has not been investigated.

Insects metabolize lipids during metamorphosis and diapause. The primary source of energy during these times is derived from the catabolism of stored lipids (Arrese & Soullages, 2010; Downer & Matthews, 1976). We hypothesized that, as energy stores become depleted in EAB pupae, there must be a limited window during which healthy beetles can be flushed from wood. Understanding the length of this window is important, as individuals of similar fitness should be used in experiments if results are to be replicable and representative of insects emerging in the wild. If insects emerging after storage were of poorer quality this would influence how we apply the results of studies estimating factors such as mortality or longevity to estimate what should be expected to occur under 'real-life' conditions.

Here, we report our observations on the fitness of EAB emerging from logs stored for 7–14 months after harvest under conditions used at our institution (i.e., initially at ambient temperatures in an enclosed structure, then at 4 °C in an environmental chamber). For our experiments we assumed that insects emerging after the shortest storage period (7 months) were of comparable condition to beetles emerging under natural conditions. We then assessed fitness for beetles exposed to increasing storage times by comparing the size, weight, fat, and water content of fresh (i.e., newly emerged) EAB, to that of EAB flushed from logs and reared under controlled conditions until death. We also assessed the effect of storage time on time to emergence and number of insects that emerged. Methods to evaporate water and procedures such as Soxhlet to solubilize and remove lipids (to obtain lean weight) have been reported in a number of insect studies (e.g., Bentz, 1999; Couvillon et al., 2011; Williams et al., 2012). However, we have found no methods for EAB. Therefore, we also developed and report methods to assess fat and water content for EAB emerging from the stored log bolts.

Material and methods

Insect collection and rearing

We harvested EAB-infested white ash, *Fraxinus americana* L., from a stand located near Exeter, ON, Canada (43.34573, –81.55772) on 22 October 2014. Logs were cut

into bolts each approximately 40 cm long and then transported to Sault Ste. Marie, ON, Canada (46.50392, –84.30557) where they were stored in an enclosed sea container on the grounds of the Great Lakes Forestry Centre (GLFC). The temperature during storage averaged -8.1 ± 8.2 °C with a maximum temperature of 14.0 °C experienced shortly after the logs were put in storage and a minimum temperature of -37.5 °C in mid-winter (Figure S1). On 24 March 2015, the logs were moved into an environmental chamber (4 °C, L0:D24) where they remained until needed. This is the standard procedure used at the GLFC since ca. 2010 to obtain EAB for experimental work. A mass collection is the most efficient option because the GLFC is located 800–1000 km from large stands of infested ash that can be harvested to obtain EAB.

At 1-month intervals between May and September 2015 groups of log bolts, with estimated average bark surface areas ranging from 0.22 to 0.25 m², were haphazardly selected from the collection in the environmental chamber and then transferred to wooden cages in a flushing room (26 °C, L16:D8). The EAB completed development in these cages which were monitored daily for emerging adults that were collected the day they were first seen in the cage. An additional set of these bolts was removed in December (after 14 months storage) and transferred to the rearing room. After emergence had completed the log bolts were dissected and examined for the presence of non-emerged EAB. All insects collected during this dissection were identified to stage using a visual guide (Chamorro et al., 2012) or by measuring the head capsule to determine the larval stage.

The newly emerged beetles were separated by sex and the first 25 male and 25 female EAB to emerge from each group of bolts were killed by freezing (-20 °C) to assess weight and fat and water content of fresh, newly emerged beetles. The next ca. 25 males and 25 females to emerge were reared until death. For rearing, up to six beetles were held in clear, 1-l plastic containers with mesh lids, given *Fraxinus uhdei* (Wenzig) leaflets as a food source and maintained in a controlled environmental chamber (27 °C, L16:D8, 60–70% r.h.). Foliage was replaced with fresh material as required. The rearing cups were checked every 24–48 h and the day of death of each beetle was recorded. All dead beetles were then collected and held at -20 °C and subjected to the same analyses as fresh beetles.

We used a micrometer to measure the widest width along the pronotum, the length of an elytron, and the length from the posterior tip of the elytron to the anterior end of the head of the beetles killed at emergence and the beetles that were reared until death ($n = \text{ca. } 100$ per storage time). We also measured the fresh weight (either at emergence or at death) to the nearest 0.1 mg using a Sartorius

1801 MPS balance. Each beetle was then dried in a drying oven (70 °C), weighed again to obtain a dry weight to the nearest 0.01 mg using a Mettler Toledo AG285 balance, subjected to a Soxhlet lipid extraction then dried again in an oven (70 °C) and weighed for a third time to obtain the lean dry weight. Details of the drying and Soxhlet lipid extraction method are given below.

Development of fat extraction protocol

A different population of EAB was used to develop the fat extraction protocols. These adult EAB were obtained from infested ash harvested from Gatineau Park, QC, Canada (45.63861, -75.95150) on 22 October 2015. Logs from these trees were transported to the GLFC and stored as above until 30 January and 5 February 2016 when they were moved to the rearing room. A total of 48 males that emerged from these bolts were placed into individual 1.5-ml microfuge tubes and killed by freezing on their day of emergence.

Previous studies that have used fat extraction methods for insects give a range of times for the various steps in the process (e.g., 1–8 days drying time; Bentz, 2006; Couvillon et al., 2011). Some studies also report that it is necessary to break the integument before drying insects (e.g., Williams et al., 2012). We tested the effect of both techniques (time and integument breaking) to determine the optimal protocol for EAB. To determine whether the intactness of the integument affected water or lipid loss in EAB adults, a blade from a pair of fine forceps was used to break an anteroposterior line in the metathorax and in the first three abdominal segments in 24 of the 48 specimens treated. To examine the effect of drying time we used the balances listed above to weigh each EAB before and after each of a series of drying times of 12, 18, 24, 36, 48, 72, 96, and 120 h. After the final drying, the specimens were subjected to Soxhlet extractions in a 500-ml combination heating mantle (Glas-Col, Terre Haute, IN, USA) set to medium heat with a cycling time of 15 min. The solvent, petroleum ether (E139; Thermo Fisher Scientific, Waltham, MA, USA), had a boiling range of 36–60 °C. Each beetle was placed into a size 00 BEEM capsule (Electron Microscopy Science, Hatfield, PA, USA). We modified each BEEM capsule by drilling 10 2-mm-diameter holes, including one hole on each end of the capsule to allow the solvent to enter the capsule and wash over the insect inside. We also engraved an identifying number onto each capsule as to permit us to identify individual beetles at the end of the extraction process.

We evaluated the effect of extraction time on the amount of lipids removed by conducting the Soxhlet procedure over a series of cumulative times (6, 12, 18, 24, 36, 48, and 72 h). As above we re-weighed each beetle after

each extraction time. Between extractions and re-weighing we dried the beetles for 12–18 h, as there was no significant difference in weight loss within this range of time periods (ANOVA: $P = 0.95$). During the first six treatment times, the beetles were placed into the cellulose Soxhlet thimble in the same order and cut and uncut specimens were distributed equally throughout the thimble. To investigate whether the location of the insect in the Soxhlet thimble influenced the amount of lipid remaining, we reversed the placement of the BEEM capsules for the last extraction so that those beetles that had always been at the bottom of the thimble were positioned at the top and vice versa. This was done because the 48 BEEM capsules filled the portion of the thimble that holds solvent and the thimble immediately siphons when the solvent reaches the top of the vessel; thus, beetles at the bottom of the thimble were almost constantly immersed in solvent but those at the top were only briefly submerged.

On the basis of the results of these trials, we determined that an initial drying time of 24 h at 70 °C was required to remove all the water from each beetle. The ideal Soxhlet extraction time was determined to be 24 h with a 15-min solvent cycle time, followed by a second drying time of 12–18 h at 70 °C to evaporate any remaining solvent. Swapping the position of the beetles in the Soxhlet thimble had no effect on the amount of lipid remaining.

Data analysis

We tested the effect of transfer date, collection time (at emergence or at death following rearing), and sex on fresh weight, fat content, and water content. For all analyses, we scaled each measure of body condition (C) (Kelly et al., 2014). Scaling C accounts for the fact that individual EAB vary in size and therefore removes the effect of body size on the measures of body condition. We took linear measures of the body length (B), elytra length (E), and thorax width (T) for all EAB and used Pearson product-moment correlation (r) to find the strongest correlation between $\log(B)$, $\log(E)$, $\log(T)$, and $\log(C)$ for each measure of body condition. We identified $\log(T)$ as the strongest correlate for the three measures of body condition (Table 1) then determined the slope (b) of the model II regression from the best-fit line of a standardized major axis regression (b_{SMA}) between $\log(T)$ and $\log(C)$.

Ideally b_{SMA} should be computed from a reference data set, independent from the experimental data (Kelly et al., 2014). However, we did not have access to a reference data set of EAB measures that contained both measures of thorax width and body condition (i.e., water content, fat content). So we compared thorax widths in the experimental data to thorax widths from a reference data set (CJK MacQuarrie, unpubl.) to determine whether thorax

Table 1 Values of Pearson's product-moment correlations between measures of body mass composition in adult emerald ash borer (all: $P < 0.001$)

Body mass composition	When collected	Sex	Body length	Elytron length	Thorax width
Fat content	At emergence	Pooled	0.5295	0.5142	0.5747
		Male	0.3839	0.3494	0.4473
		Female	0.5883	0.5844	0.6293
	At death	Pooled	0.6444	0.6530	0.6548
		Male	0.5414	0.5676	0.5763
		Female	0.6551	0.6526	0.6486
Fresh weight	At emergence	Pooled	0.9618	0.9551	0.9667
		Male	0.9354	0.9267	0.9394
		Female	0.9687	0.9639	0.9734
	At death	Pooled	0.8939	0.8923	0.8984
		Male	0.8361	0.8376	0.8526
		Female	0.9114	0.9072	0.9114
Water content	At emergence	Pooled	0.9460	0.9400	0.9473
		Male	0.9091	0.9027	0.9093
		Female	0.9562	0.9515	0.9558
	At death	Pooled	0.8108	0.8038	0.8083
		Male	0.7944	0.8008	0.8038
		Female	0.8093	0.7955	0.8075

widths in the experimental data were representative. These values differed by 0.144 mm (reference: 2.86 ± 0.29 mm, $n = 439$; this experiment: 2.72 ± 0.33 mm, $n = 592$; $t = 7.251$, d.f. = 1 029, $P = 8 \times 10^{-13}$), which means that the measures of b_{SMA} that we computed should be interpreted with caution as they may be influenced by our experimental treatments. Once we had determined a b_{SMA} value it was used in the equation:

$$\hat{C}_i = C_i \left[\frac{T_0}{T_i} \right]^{-b_{\text{SMA}}},$$

where C_i is the body condition measure for each individual (i), T_0 is the arithmetic mean of thorax width (2.72 mm), T_i is the thorax width for each individual, and \hat{C}_i is the predicted (i.e., scaled) measure of body condition. The resulting values, \hat{C}_i , were what we used as the response variable in the subsequent analyses.

We also tested the effect of transfer date and sex on the number of days until emergence occurred and the number of insects that emerged from the logs. To test the effect of transfer date and sex on time to emergence, we first fit a parametric model to the data. However, we were not able to satisfy the assumptions of the parametric test so we instead fit a non-parametric Cox proportional hazards model to the emergence data with 'sex' and 'transfer date' as the predictor variables. This analysis had the advantage of allowing us to include data from the unemerged insects to determine estimates of time to emergence. For this

analysis, we assumed the sex ratio of the non-emerged insects to be 50:50. Total emergence in this experiment was estimated for each emergence box, which sometimes were provisioned with different numbers of logs so to test for the number of insects that emerged in each box we used the number of bolts as a covariate in the statistical models. Finally, we also examined whether the number of insects that failed to emerge from the logs, and the stage of the non-emerged insects, was influenced by transfer date.

All statistical analyses were done in the R statistical computing language v.3.4 (R Development Core Team, 2017). The values for \hat{C}_i , were analyzed using linear models, which we examined for issues with heterogeneity or missing co-variables. The data for fat content, emergence, and the number of insects not emerging all exhibited heterogeneity in the residuals for one or both of the main effects. We resolved this by applying a covariance function. We tested for the significance of the main effects using ANOVA. The Cox proportional hazards model was fit and tested using procedures from Harrell (2015). All analysis code and the raw data are deposited on the Dryad data repository (Fick et al., 2018).

Results

Emergence

The number of EAB adults that emerged from the logs was influenced by the length of time for which the bolts were stored, the sex of the emerging beetles, and the number of

logs in the emergence cage (Table 2, Figure S2). More males than females emerged, fewer insects emerged for the longer than for the shorter storage times (≥ 10 vs. < 10 months) and more insects emerged into cages with more logs (Figure 1).

The number of days until emergence was also influenced by sex and the storage duration of the logs (Table 3, Figure S3). However, these effects were weak. Emergence began between 21 and 25 days after logs were placed in the emergence cages, with 50% of all emergence having occurred by 30 days. The exception was those logs that were placed into the cages in December (i.e., after 14 months). Emergence from those logs began later (around 32 days), with 50% emergence having completed by around 37 days. In all cohorts males emerged a few days before females (Figure 2).

A total of 281 samples were extracted from the logs after emergence had completed, or an average (\pm SD) of 3.9 ± 0.9 samples per log. All larval stages of EAB were represented in the non-emerged insects, with the exception of first instars. First instar EAB are so small that they are difficult to see without the aid of a microscope and could therefore have been missed. Twelve percent of the samples ($n = 36$) could not be identified to stage (e.g.,

Table 2 Results of Wald's F-test for significance of the main effects — month logs were transferred, sex of emerald ash borer (EAB), and the number of logs in a rearing box — on three response variables. Significance is tested by sequentially adding model terms; 'Intercept' represents the significance of a model with no main effects

Response	Term	d.f.	F	P
No. emerged EAB	Intercept	1	956.5	2.2×10^{-39}
	Month	5	15.2	9.3×10^{-10}
	Sex	1	12.9	6.3×10^{-4}
	No. logs	1	6.46	1.4×10^{-2}
No. EAB samples in bolts	Intercept	1	351.1	2.2×10^{-17}
	Month	5	2.23	7.9×10^{-2}
	No. logs	1	31.2	5.6×10^{-6}
No. overwintered EAB samples in bolts	Intercept	1	289.6	2.7×10^{-16}
	Month	5	0.962	4.6×10^{-2}
	No. logs	1	5.62	2.5×10^{-2}

because only a piece of the insect was recovered) or were not definitively determined to be EAB. These samples were not included in our analyses. Storage duration of the logs

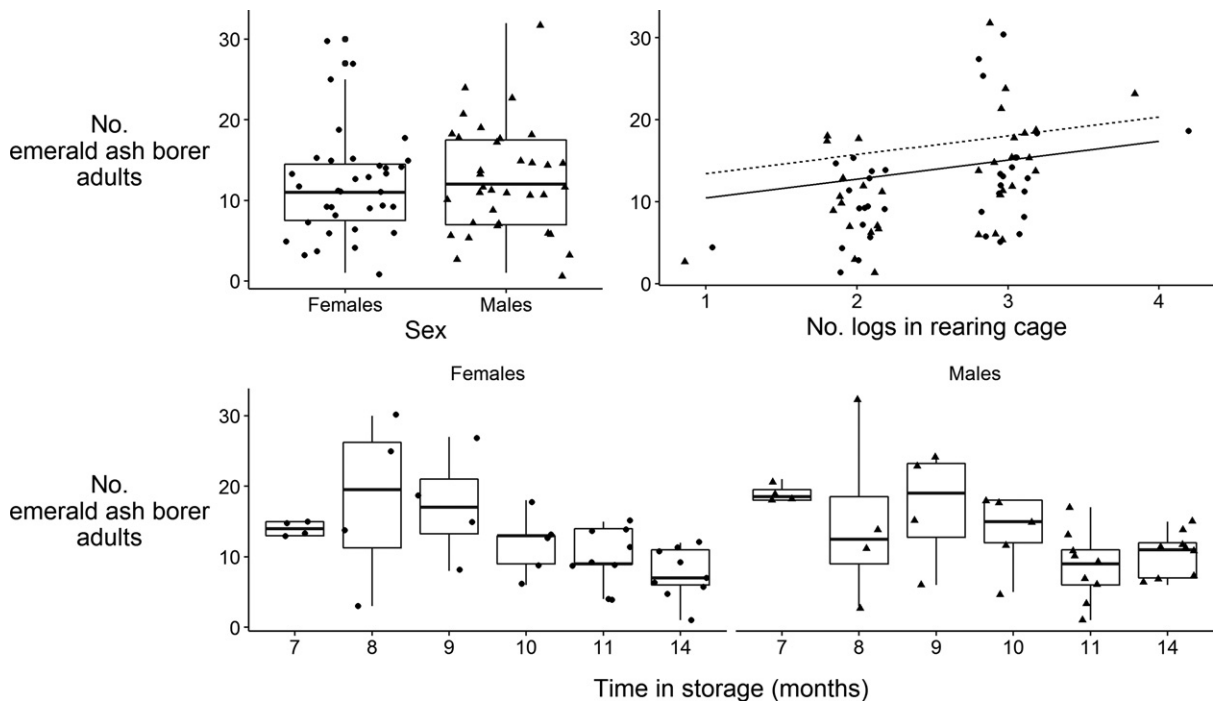


Figure 1 Number of emerald ash borer (EAB) adults emerging from infested ash bolts after a period of storage. More EAB males (triangles, broken line) emerged than females (dots, solid line) (upper left panel), and in cages with more bolts (upper right panel). Overall emergence was higher in bolts stored for less than 10 months compared to bolts stored for 10 months or longer for both males and females (lower panels). Box and whisker plots show the median (horizontal line), the 25th and 75th percentiles (box) and $1.5 \times$ the interquartile range (whiskers). All panels show the raw data (points) which have been offset to reduce visual overlap.

Table 3 Results of analysis of deviance test for the significance of the main effects — month logs were transferred, sex of emerald ash borer (EAB) — on two response variables. Significance is tested by sequentially adding model terms; ‘Null model’ represents the significance of a model with no main effects

Response	Term	d.f.	Log likelihood	χ^2	P	
Cox proportional hazards for emergence of EAB	Null model		−5403.02			
	Sex	1	−5401.32	3.41	6.5×10^{-2}	
	Month	5	−5338.23	126.16	$<2 \times 10^{-16}$	
	Sex:month	5	−5335.93	4.60	4.7×10^{-1}	
	Term	d.f.	Deviance	Residual d.f.	Residual deviance	P
Developmental stage of EAB recovered from logs	Null model			244	315.03	
	Month	5	23.05	239	291.98	3.3×10^{-4}

had no effect on the number of insects that failed to emerge, but the number of logs that were in each flushing cage had a positive effect on the number of samples that were recovered (Table 2). Of all the samples that could be identified, 37% ($n = 104$) were in the fourth instar or younger and would have required additional feeding and a

period of overwintering to complete development to the adult stage (Cappaert et al., 2005; Tluczek et al., 2011). Thus, these insects were not able to emerge in our experiments. We tested whether the length of time in storage influenced the probability of a sample being older or younger than fourth instar (i.e., overwintered or not). Our

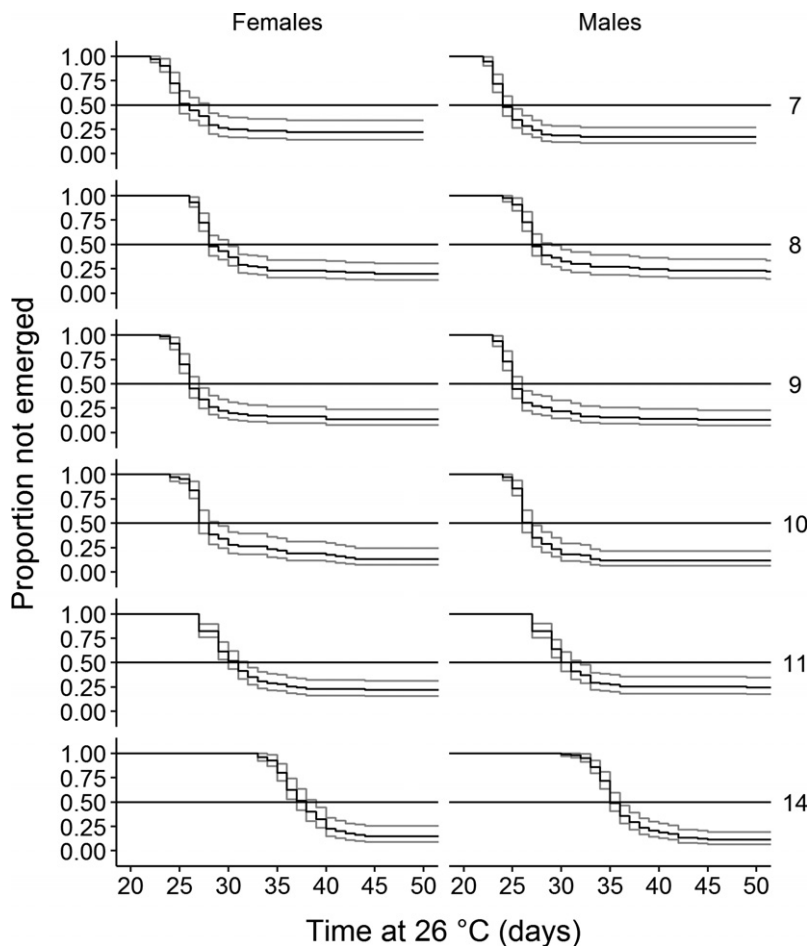


Figure 2 Emergence proportion of emerald ash borer (EAB) adults from white ash logs, after storage for 7–14 months, followed by transfer to an emergence cage at 26 °C. Male EAB (right column) emerge earlier than female EAB (left column), and those stored for 14 months (bottom row) began emerging later than insects stored for shorter periods (upper rows). Black lines show model estimates of the proportion of the total populations still not emerged at a given day, with estimate error in grey lines.

Table 4 b_{SMA} values for measures of body condition in emerald ash borer

Body mass composition	When collected	Sex	Month	b_{SMA} (min, max)
Fat content	At emergence	Female	May	6.497 (5.009, 8.428)
			June	6.645 (5.172, 8.539)
			July	6.561 (4.563, 9.435)
			August	4.9 (3.355, 7.158)
			September	8.562 (5.898, 12.428)
			December	4.528 (3.32, 6.175)
		Male	May	10.229 (7.34, 14.254)
			June	5.642 (3.942, 8.074)
			July	6.695 (5.12, 8.756)
			August	7.666 (5.405, 10.874)
			September	7.096 (4.744, 10.614)
			December	5.424 (3.669, 8.019)
	At death	Female	May	4.822 (3.428, 6.785)
			June	5.708 (4.276, 7.619)
			July	3.258 (2.4, 4.424)
			August	2.835 (2.095, 3.837)
			September	4.033 (2.883, 5.642)
			December	3.692 (2.719, 5.013)
		Male	May	4.373 (3.173, 6.026)
			June	5.304 (3.634, 7.741)
			July	4.342 (3.441, 5.478)
			August	3.601 (2.506, 5.176)
			September	3.729 (2.606, 5.336)
			December	4.071 (2.716, 6.101)
Fresh weight	[All]	[All]	[All]	3.012 (2.885, 3.145)
Water content	[All]	[All]	[All]	3.491 (3.293, 3.701)

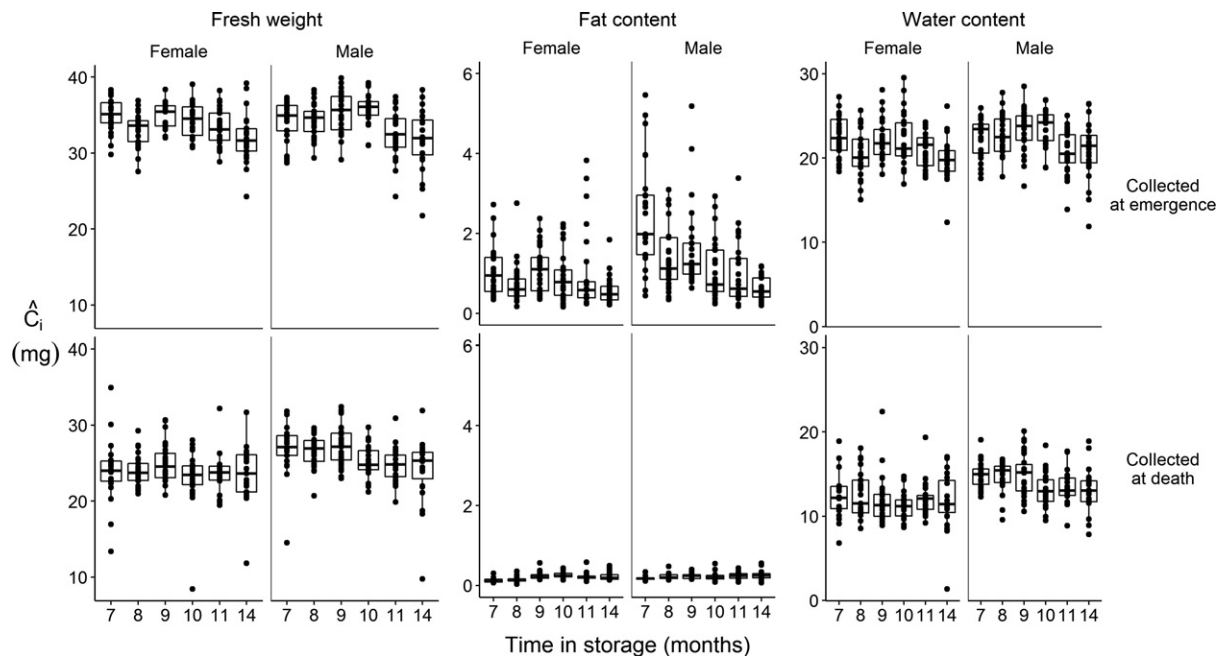
**Figure 3** Body composition values (fresh weight, fat and water content; all mg) of male and female emerald ash borer (EAB) adults from white ash logs, after storage for 7–14 months, collected at emergence or at death. See Figure 1 for an explanation of box and whisker plots.

Table 5 Results of Wald's F-test for the significance of the main effects — month logs were transferred, if the emerald ash borers (EAB) were collected at death or at emergence — on three scaled measures of body condition (fat content, fresh weight, and water content) in male and female EAB. Significance is tested by sequentially adding model terms; 'Intercept' represents the significance of a model with no main effects

Response	Sex	Term	d.f.	F	P
Fat content	Males	Intercept	1	1222.3	6.7×10^{-105}
		When collected	1	232.9	8.0×10^{-39}
		Month	5	1.748	1.2×10^{-1}
		When collected:month	5	13.58	6.8×10^{-39}
	Females	Intercept	1	985.5	3.1×10^{-93}
		When collected	1	181.0	4.2×10^{-32}
		Month	5	12.35	7.9×10^{-11}
		When collected:month	5	4.857	2.8×10^{-4}
Fresh weight	Males	Intercept	1	19050.8	6.1×10^{-268}
		When collected	1	339.6	7.6×10^{-51}
		Month	5	3.956	1.7×10^{-3}
		When collected:month	5	2.861	1.5×10^{-2}
	Females	Intercept	1	27298.3	6.6×10^{-282}
		When collected	1	840.8	1.9×10^{-86}
		Month	5	4.482	6.0×10^{-4}
		When collected:month	5	2.861	1.5×10^{-2}
Water content	Males	Intercept	1	7212.5	6.7×10^{-208}
		When collected	1	344.5	2.4×10^{-51}
	Females	Intercept	1	10567.9	1.2×10^{-222}
		When collected	1	840.3	7.1×10^{-86}
		Month	5	1.796	1.1×10^{-1}
		When collected:month	5	3.228	7.5×10^{-3}

results show that there was an effect (Table 3). However, for some storage periods the effect was positive (i.e., most individuals were old enough to have been able to emerge), whereas in other periods the effect was negative (i.e., most individuals were not old enough to have been able to emerge) (Figure S4). When we repeat our analysis from above but considering just those insects that could have emerged (i.e., those that had overwintered), we obtained the same result (Table 2).

Body condition

Thorax width had the highest correlation with all three body condition measures (Table 1; Figure S5). There was no effect of emergence time, sex, or collection time on the b_{SMA} values for fresh weight or water content so we computed one b_{SMA} for each measure of body condition (Table 4). The value of b_{SMA} for fat content was influenced by all three main effects, so we computed separate b_{SMA} values for each combination of these factors (Table 4). The resulting scaled body condition measures, \hat{C}_i , lacked the linear trend seen in the raw data (Fick et al., 2018).

Our first analysis of the body condition data resulted in statistical interactions between sex and one or more of the other main effects (Fick et al., 2018). These interactions were difficult to interpret. Therefore, we opted to split the

analyses by sex because female EAB are already known to be larger than male EAB, making sex the least interesting of the main effects.

The fresh weight of males and females was influenced by both when the insects were collected (i.e., at emergence or at death) and the length of time for which the logs were stored (Figure 3). For both males and females, fresh weight was highest in EAB that emerged from the July and August cohorts of bolts. However, fresh weight at death was the same for all females, and only slightly higher for males in the earlier cohorts. This resulted in a statistically significant interaction between the two main effects for females, but not for males (Table 5, Figure S6).

The fat content of males and females was also influenced by when the insects were collected and the length of time the logs were stored (Figure 3). Fat content of males was highest in the early cohort of logs and declined in later sets, whereas fat content in females was the same among the first three or four cohorts and then declined. Both sexes had essentially no fat left at death. This pattern resulted in a statistically significant interaction between the main effects for both sexes (Table 5, Figure S6).

The water content of female EAB was influenced by both when the insects were collected and how long the logs were stored. The water content of male EAB was only

influenced by when the insects were collected (Figure 3). In females, water content was highest among the early cohorts of logs. Water content in both sexes was lower at death than at emergence. This pattern resulted in a significant interaction between the main effects for female insects, but only a significant effect of collection time for males (Table 5, Figure S6).

Discussion

Long-term storage of EAB-infested logs appears to influence the overall number of insects that can be produced from infested logs but has few effects on the subsequent fitness of emerging insects. This failed to support our hypothesis that the quality of insects should decline with increased time, and failed to support anecdotal observations that insects emerging from long-term storage are less viable. However, our results do support anecdotal observations that older logs tend to produce fewer beetles. These findings also suggest that EAB can survive long periods in infested wood if kept at temperatures below its emergence threshold (i.e., below 10 °C; see: Brown-Rytlewski & Wilson, 2004). We also find a weak but significant effect of sex on time to emergence, which suggests that EAB exhibits protandry (i.e., male emergence occurs before female emergence). In previous experiments, we have observed that males seem to emerge 1 or 2 days before females but this is the first time the phenomenon has been quantified.

Other buprestid beetles have been reported to survive and emerge from wood after long periods (e.g., Every & Rudinsky, 1962). Smith (1962) documented 32 reports of *Buprestis* species found in, or emerging from, wood incorporated into structures, which suggests the phenomenon of prolonged development is common in the Buprestidae. Live larvae and adults were found in wood in structures (mostly homes) between 12 and 51 years after those structures had been built (larval mean \pm SD = 22 ± 13 years, $n = 12$; adult mean = 22 ± 12 years, $n = 17$) (Fick et al., 2018). More recent experiments have examined short (i.e., 24–48 h) cold-storage period on quality and flight performance of adult insects (Chubaty et al., 2014; Matveev et al., 2017), but ours is the first study to examine the effect of long-term storage of infested wood on the quality of the insects that subsequently emerge. Although given the extreme cases presented in Smith (1962), our data may be more aptly classified as short-term storage as well.

The goal of the extraction trials was to determine three things: (1) the length of time required to remove water and lipid from the beetles, (2) whether cutting the exoskeleton increased the amount of water and lipid loss,

and (3) whether the position in the Soxhlet thimble affected the amount of lipid loss. We observed no decrease in water or lipid loss after 24 h of exposure time which is consistent with times reported for other insects. For example, fat extraction times of 8–12 h were required for *Dendroctonus ponderosae* (Hopkins) and *Ips paraconfusus* Lanier (both Curculionidae) (Bentz, 2006; Hagen & Atkins, 2009), whereas an extraction time of 72 h was required for two species of *Pterostichus* (Carabidae) (Ostman, 2005). Fat extraction times appear to roughly scale with body size, as adult EAB are larger than *D. ponderosae* and *I. paraconfusus*, and smaller than adult *Pterostichus*. The average lipid content of EAB in our experiment (7.7%) also appears to be below the average of 14% for Coleoptera (Lease & Wolf, 2011). Breaking the integument (e.g., Couvillon et al., 2011) had no effect on the resulting fat extracted, nor did changing the position of the insects in the Soxhlet thimble. That these steps are not needed simplifies the process for this procedure. In our experiments, we selected BEEM capsules (1.0 ml) over the larger microfuge tubes (1.5 ml), because this permitted us to process more insects in a single Soxhlet thimble while still accommodating the largest EAB that we collected. In addition, the length of a microfuge tube was similar to the diameter of the thimble and this led to tubes becoming stuck during early trials. Using BEEM capsules resolved this issue.

Examining the emergence of adult EAB in our experiments revealed an interesting pattern. Logs that had been stored for more than 10 months produced fewer insects than those that had been stored for less than 10 months. However, dissection of the logs at the end of the experiment showed that all logs had approximately the same number of EAB at the start of the experiment. Thus, it would appear that storage does influence the ability of EAB to successfully pupate and emerge leading to the reduction in overall emergence that we observed after 10 months of storage. However, this 'storage effect' does not seem to influence the quality or fitness of the subsequent adult beetles. We did notice a decrease in the longevity of individuals from the longest storage periods. Insects from most of the storage periods tended to live ca. 25–30 days in captivity, but those that were emerged after 11 and 14 months lived 15–25 days. However, insects emerging in the later storage periods were emerging in the late fall and early winter (September and December) at the GLFC. We have noticed that the quality of our greenhouse-grown *F. uhdei* ash foliage tends to decrease during this period, possibly associated with senescence triggered by reduced light levels during the northern hemisphere autumn. Thus, decreased lifespan may be an effect of storage or a change in food quality, or both. Our inability to

control for these effects is why we did not compare lifespan or fecundity in this study.

The effect of storing insects for long periods may select for individuals that were of greater quality before pupating. If this hypothesis is true, we might expect higher variability in the body condition of insects that were stored for shorter periods because low-quality individuals would not have been removed from the sample (by mortality). With increased storage time, less fit individuals may be eliminated thus reducing the variability within the population of later emerging insects. If we examine the fat content of male EAB at emergence we see a pattern of greater variance in that of beetles that emerged in May vs. those that emerged in December. This pattern supports our hypothesis. However, it is the only measure of body condition that does.

It is assumed that EAB was introduced to North America on infested wood or wood products (Cappaert et al., 2005; Herms & McCullough, 2014; Siegert et al., 2014). However, no study has determined whether EAB can survive in wood during a typical period of shipment from the putative home range in eastern Asia (Bray et al., 2011; Keever et al., 2012) to the North American epicentre in Detroit, MI, USA – Windsor, ON, Canada (Haack et al., 2002). We show that EAB can survive a period of storage and transit of at least 14 months if they are maintained below their emergence threshold (e.g., under refrigeration) and still emerge as adults with no effect on their viability. However, these results also show that emergence times under the right conditions are shorter (e.g., 21 days at 26 °C) than a typical sea voyage from the home range to the epicenter (35–40 days). Thus, pupal EAB transported under ambient conditions during the northern hemisphere summer would likely emerge in transit and not at the destination. Work et al. (2005) showed that refrigerated cargo presented the greatest risk for introductions of species to the USA. Our findings are consistent with this observation and suggest the initial EAB introduction to North America occurred via infested wood that was brought to North America during the northern hemisphere winter or on infested wood material that was kept under refrigeration during transit. Moreover, to have contained viable EAB adults this infested material must have been transported during the year after the tree was harvested.

Emerald ash borer adults can be obtained from infested logs stored for over a year. These adults retain the same viability and apparent fitness as insects that emerge after a period of storage typical to that of an EAB in a standing tree. However, the number of insects that emerge does decrease with length of storage meaning that more infested material is required to produce the same number of EAB.

Rearing programs for biological control agents and research programs can be confident that EAB obtained from stored logs should exhibit the same quality regardless of the storage period. However, our results also show that it is necessary to increase the amount of wood that is reared as storage time increases. Research programs that require high-quality larvae (e.g., for rearing larval parasitoids) should be cautious when using EAB obtained from stored logs as these insects may no longer be viable. Our results also show that EAB that emerge from infested wood into new areas are not affected by any developmental delay they may have experienced during transport. Although the absolute number of beetles emerging, and thus the probability of establishing a new infestation (Drake & Lodge, 2006), is lower for logs that have been subject to an artificial storage.

Acknowledgements

We thank J Allard, K Boissoneau, M Gray, G Jones, Y Liu, D Lombardo, G Roth, K Scarfone, R Scharbach, T Sharma, J Skillings, and A Wardlaw for field and laboratory assistance with this project.

References

- Arrese EL & Soulages JL (2010) Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* 55: 207–225.
- Bentz BJ (1999) Variation in two life history traits of *Dendroctonus ponderosae* from lodgepole and ponderosa pines in Idaho and Utah. *Proceedings of a Workshop on Bark Beetle Genetics: Current Status of Research* (ed. by JL Hayes & K Raffa), pp. 28–29. USDA, Forest Service, Pacific Northwest Research Station General Technical Report PNW-GTR-466, Portland, OR, USA.
- Bentz BJ (2006) Mountain pine beetle population sampling: Inferences from Lindgren pheromone traps and tree emergence cages. *Canadian Journal of Forest Research* 36: 351–360.
- Bray AM, Bauer LS, Poland TM, Haack RA, Cognato AI & Smith JJ (2011) Genetic analysis of emerald ash borer (*Agrilus planipennis* Fairmaire) populations in Asia and North America. *Biological Invasions* 13: 2869–2887.
- Brown-Rytlewski DE & Wilson MA (2004) Tracking the emergence of emerald ash borer adults. *Emerald Ash Borer Research and Technology Development Meeting* (ed. by V Mastro & R Reardon), pp. 13–14. USDA, Forest Service publication FHTET-2004-15, Morgantown, WV, USA.
- Cappaert D, McCullough DG, Poland TM, Siegert NW & Seiger NW (2005) Emerald ash borer in North America: a research and regulatory challenge. *American Entomologist* 51: 152–165.
- Chamorro ML, Volkovitch MG, Poland TM, Haack RA & Lingafelter SW (2012) Preimaginal stages of the emerald ash borer,

- Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae): an invasive pest on ash trees (*Fraxinus*). PLoS ONE 7: e33185.
- Chen YG & Poland TM (2009) Biotic and abiotic factors affect green ash volatile production and emerald ash borer adult feeding preference. *Environmental Entomology* 38: 1756–1764.
- Chubaty AM, Hart M & Roitberg BD (2014) ‘To tree or not to tree’: the role of energy limitation on host tree acceptance in a bark beetle. *Evolutionary Ecology Research* 16: 337–349.
- Couvillon MJ, Jandt JM, Bonds J, Helm BR & Dornhaus A (2011) Percent lipid is associated with body size but not task in the bumble bee *Bombus impatiens*. *Journal of Comparative Physiology A* 197: 1097–1104.
- Downer RGH & Matthews JR (1976) Patterns of lipid distribution and utilisation in insects. *American Zoologist* 16: 733–745.
- Drake JM & Lodge DM (2006) Allee effects, propagule pressure and the probability of establishment: risk analysis for biological invasions. *Biological Invasions* 8: 365–375.
- Every RW & Rudinsky JA (1962) The Golden Buprestid: A Wood Boring Beetle. Oregon State University Extension Circular 713, OSU, Corvallis, OR, USA.
- Fick WE & MacQuarrie CJK (2018) Data from: An artificial delay in emergence influences the number but not the fitness of adult emerald ash borer emerging from infested ash wood. Dryad Digital Repository (www.datadryad.org).
- Haack RA, Jendek E, Liu H, Marchant KR, Petrice TR et al. (2002) The emerald ash borer: a new exotic pest in North America. *Newsletter of the Michigan Entomological Society* 47: 1–5.
- Hagen BW & Atkins MD (2009) Between generation variability in the fat content and behavior of *Ips paraconfusus* Lanier. *Zeitschrift für Angewandte Entomologie* 79: 169–172.
- Harrell FE (2015) *Regression Modeling Strategies*. Springer, New York, NY, USA.
- Herms DA & McCullough DG (2014) Emerald ash borer invasion of North America: history, biology, ecology, impacts, and management. *Annual Review of Entomology* 59: 13–30.
- Keever CC, Nieman C, Ramsay L, Ritland CE, Bauer LS et al. (2012) Microsatellite population genetics of the emerald ash borer (*Agrilus planipennis* Fairmaire): comparisons between Asian and North American populations. *Biological Invasions* 15: 1537–1559.
- Kelly CD, Tawes BR & Worthington AM (2014) Evaluating indices of body condition in two cricket species. *Ecology and Evolution* 4: 4476–4487.
- Lease HM & Wolf BO (2011) Lipid content of terrestrial arthropods in relation to body size, phylogeny, ontogeny and sex. *Physiological Entomology* 36: 29–38.
- Matveev E, Kwon JJ, Judd GJR & Evenden ML (2017) The effect of cold storage of mass-reared codling moths (Lepidoptera: Tortricidae) on subsequent flight capacity. *Canadian Entomologist* 149: 391–398.
- Orlova-Bienkowskaja MJ (2015) Cascading ecological effects caused by the establishment of the emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae) in European Russia. *European Journal of Entomology* 112: 778–789.
- Ostman O (2005) Asynchronous temporal variation among sites in condition of two carabid species. *Ecological Entomology* 30: 63–69.
- R Development Core Team (2017) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Siebert NW, McCullough DG, Liebhold AM & Telewski FW (2014) Dendrochronological reconstruction of the epicentre and early spread of emerald ash borer in North America. *Diversity and Distributions* 20: 847–858.
- Smith DN (1962) Prolonged larval development in *Buprestis aurulenta* L. (Coleoptera: Buprestidae). A review with new cases. *Canadian Entomologist* 94: 586–593.
- Timms LL, Smith SM & de Groot P (2006) Patterns in the within-tree distribution of the emerald ash borer *Agrilus planipennis* (Fairmaire) in young, green-ash plantations of south-western Ontario, Canada. *Agricultural and Forest Entomology* 8: 313–321.
- Thuczek AR, McCullough DG & Poland TM (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.
- Ulyshen MD, Duan JJ & Bauer LS (2010) Interactions between *Spathius agrili* (Hymenoptera: Braconidae) and *Tetrastichus planipennisi* (Hymenoptera: Eulophidae), larval parasitoids of *Agrilus planipennis* (Coleoptera: Buprestidae). *Biological Control* 52: 188–193.
- USDA (United States Department of Agriculture) (2017) Cooperative Emerald Ash Borer Project. Initial County EAB Detections in North America (www.emeraldashborer.info) (accessed on 1/11/2017)
- Williams C, Hellmann J & Sinclair B (2012) Lepidopteran species differ in susceptibility to winter warming. *Climate Research* 53: 119–130.
- Work TT, McCullough DG, Cavey JF & Komsa R (2005) Arrival rate of nonindigenous insect species into the United States through foreign trade. *Biological Invasions* 7: 323–332.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maximum, minimum, and average temperatures experienced by logs during storage. The blue line is the loess-derived moving average temperature during the storage period \pm SE (grey band), the horizontal line indicates 4 °C, or the indoor storage temperature (shown for comparison).

Figure S2. Model coefficients for generalized least squares fit of sex and months in storage for emerald ash borer emergence.

Figure S3. Coefficients of the Cox proportional hazards model for emergence of emerald ash borer from infested logs.

Figure S4. Total abundance of emerald ash borer larvae — fourth instar or younger, and older than fourth instar — recovered from ash logs after emergence had stopped.

Figure S5. Correlation of body measures and body condition for emerald ash borer by sex and when insects were collected.

Figure S6. Model coefficients for linear or generalized least squares fit of months in storage and time of collection for emerald ash borer body composition models.