Bulletin of Insectology **70** *(1): 91-99, 2017*
ISSN 1721-8861

An artificial diet for rearing three exotic longhorn beetles invasive to Europe

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Abstract

Anoplophora glabripennis, *Anoplophora chinensis* and *Psacothea hilaris hilaris* are three invasive exotic longhorn species (Coleoptera Cerambycidae) threatening native broadleaf trees in Europe and North America. Field studies on invasive species are somewhat difficult in the areas of introduction due to the application of eradication measures and the activation of quarantine protocols. Rearing these species in standard laboratory conditions would allow specific ecological and biological investigations to be conducted. In this paper, the rearing of these longhorn beetles has been tested on an artificial diet in laboratory conditions. The tested diet can be used to obtain viable healthy adults of each of the three studied species. *P. h. hilaris* had the best rearing performance with 74% of eggs producing new adults, while *A. chinensis* and *A. glabripennis* were poorer with 24.7% and 23.3%, respectively. The low percentage of emerging *A. glabripennis* and *A. chinensis* adults was due mainly to a high mortality of their first instar larvae not entering the diet. Moreover, *A. chinensis* and *A. glabripennis* had a mean development time, 60.06 and 37.29 weeks, respectively (including the chilling periods required for pupation), longer than *P. h. hilaris* (16.1 weeks). During development, larval moults varied according to species and within species ranging from 5-7 (*P. h. hilaris*), 6-11 (*A. chinensis*) and 7-8 (*A. glabripennis*) moults, respectively. Adults of *A. glabripennis* and *P. h. hilaris* reared on the diet were bigger than wild specimens collected from the same population, whereas *A. chinensis* adults were smaller. Adult survival was shorter in *A. glabripennis* (62.9 days) than in *P. h. hilaris* (119.3 days). According to the different performance of the three species, the rearing costs were about 2.0, 8.1 and 16.1 US dollars per adult beetle for *P. h. hilaris*, *A. glabripennis* and *A. chinensis*, respectively. A laboratory insect population has to be cost effective and self-sustainable over time, and the tested diet provided valuable results for the low-cost mass rearing of these invasive longhorn beetles.

Key words: *Anoplophora glabripennis*, *Anoplophora chinensis*, *Psacothea hilaris hilaris*, laboratory rearing, exotic species, ALB, CLB, YLB.

Introduction

Global trade and climate change facilitate the introduction, establishment and spread of many invasive exotic species (Meyerson and Mooney, 2007; Jucker and Lupi, 2011). Because international trade is quickly increasing in volume and becoming faster, biological invasions are expected to grow exponentially in the near future (Hulme, 2009). Wood boring beetles, including longhorn beetles (Coleoptera Cerambycidae), are one of the most successful groups of invaders. They are easily transported in timber and wood packaging materials, escaping phytosanitary survey and protected from adverse climatic conditions during the trip from their native countries (Brockerhoff *et al.*, 2006; Haack, 2006; Rassati *et al.*, 2014; 2015). International movement of invasive wood boring beetles hence represents a severe threat to forests worldwide (Holmes *et al.*, 2009; Vilà *et al.*, 2009; Gandhi and Herms, 2010).

The Asian Longhorn Beetle (ALB) *Anoplophora glabripennis* (Motschulsky), the Citrus Longhorn Beetle (CLB) *Anoplophora chinensis* (Forster), and the Yellow-Spotted Longicorn Beetle (YLB) *Psacothea hilaris hilaris* (Pascoe) are three Asian flat-faced longhorn species invasive to Europe and North America. ALB and CLB are highly polyphagous insects developing in a large number of broadleaf species growing in urban parks and gardens (Hérard *et al.*, 2006; Haack *et al.*, 2010), and are recognized as the most destructive wood borers introduced into North America and Europe in the last decades (Jucker *et al.*, 2006; Hu *et al.*, 2009). Instead, YLB develops only in woody species of the Moraceae family (Kojima and Nakamura, 1986; Basset *et al.*, 1997; Shintani *et al.*, 2003), affecting sericulture by killing mulberry trees (*Morus* spp.) cultivated to rear silkworm, *Bombyx mori* (L.), with serious economic damage in the native regions (Iba and Shiokawa, 1990; Shintani and Ishikawa, 1997). YLB has been recorded in Europe since 2005, in Northern Italy (Jucker *et al.*, 2006; Lupi *et al.*, 2013), where it mainly infests fig trees (*Ficus carica* L.) and very rarely mulberry trees. Fig is one of the most characteristic and important tree species of the whole Mediterranean basin, and the establishment of this exotic pest is a serious new economic and ecological threat.

The major damage caused by these three invasive exotic species is related to the feeding activity of the larvae, which, following a first short period spent in the phloem, bore long tunnels deeply in the xylem of trunk, branches and roots of live plants (Haack *et al.*, 2010; Lupi *et al.*, 2013). Infested trees progressively decline and die within a few years. Before mating and laying eggs in the phloem of healthy trees, newly emerged immature adults feed for a few weeks on leaves and

tender bark of twigs to reach sexual maturation (Haack *et al.*, 2010). The whole life-cycle can be completed in 1-3 years depending on host species, latitude and local climatic conditions (Adachi, 1994; Shintani and Ishikawa, 1997; Sabbatini Peverieri and Roversi, 2010; Faccoli *et al.*, 2015; Straw *et al.*, 2015).

Although many ecological and biological observations were focused on ALB, CLB and YLB in recent years (Hu *et al.*, 2009; Haack *et al.*, 2010; and literature therein) the possibility of conducting scientific investigations was often hindered by the breeding traits of these species. Although mass rearing is necessary to perform specific researches requiring a large number of insects annually (Keena, 2005), the rearing of exotic wood boring species is very difficult for many reasons (Petersen-Silva *et al.*, 2014). First, their status as quarantine species prevents the movement of insects and infested materials outside the infestation area. Second, the long time required by larvae to develop into new adults makes rearing very complex to perform in naturally infested logs and branches, as once cut and taken to the laboratory they dry out in a few months causing large larval mortality (Hanks *et al.*, 1999). Third, larval development occurs deep in the wood making direct biological observations impossible, and affecting the possibility of larval collection from the wood. Lastly, the lack of specific aggregation pheromones available for ALB, CLB and YLB reduces the possibility of catching large numbers of adults to use in specific rearing or trials. All these characteristics limit insect availability and related investigations.

Insect rearing on an artificial diet is hence a topic that deserves exploration (e.g., Vanderzant, 1974; Cohen, 2015). Some specific diets have already been developed for longhorn beetles. Harley and Wilson (1968), for instance, developed the first specific diet for a longhorn beetle *Plagiohammus spinipennis* (Thomson), a species from the same subfamily (Lamiinae) as ALB, CLB and YLB. A specific diet for ALB was recently proposed by Zhao *et al.* (1998), then modified by Dubois *et al.* (2002), allowing a good larval development but, due to the low moisture content and rapid solidification, the diet was not easy to dispense and extremely expensive. As suggested by Cohen (2015), a diet efficient for mass rearing should be cheap, easy to prepare and also quickly dispensable in high volume. The diet proposed by Keena (2005) for ALB was the most suitable according to these parameters, even if the estimated rearing costs resulted as being very expensive. Instead, mass rearing of CLB was tested on citrus trees during experiments carried out in Japan (Adachi, 1994) and on artificial diet in preliminary assays (Murakoshi, 1981). Although artificial diets already exist for ALB and CLB, they are usually expensive, difficult to prepare and dispense, and in some case poorly effective (Murakoshi, 1981).

In previous preliminary studies, YLB was reared using a silkworm diet (Fukaya and Honda, 1992; Fukaya *et al.*, 1996), as mulberry is one of the main host plants for this beetle. Another mulberry-based diet used for silkworm (Cappellozza *et al.*, 2005) was recently tested for the artificial rearing of *P. h. hilaris* giving promising results (Lupi *et al.*, 2015b). In this respect, YLB may be used as model species to define a rearing protocol on an artificial diet suitable for other exotic longhorn beetles. As mulberry tree is a host species common to ALB, CLB and YLB, the goal of this study is to investigate the possible use of a cheap mulberry-based artificial diet previously created for the silkworm (Cappellozza *et al.*, 2005) to obtain a permanent ALB, CLB and YLB laboratory rearing. The main aims are to assess 1) diet acceptance by larvae, 2) rearing performance in terms of larval survival and developmental time, 3) size and longevity of the emerged adults, and 4) cost effectiveness of the tested diet in comparison with other commercially available diets.

Materials and methods

Adult and egg sampling

ALB. In spring 2013, ALB infested logs were collected from the infestation area at Cornuda in North East Italy (45°49'N 12°00'E) (Faccoli *et al.*, 2011; 2015; 2016; Favaro *et al.*, 2015) and stored in metal net cages (2 × 1 × 1 m) in outdoor conditions until adult emergence. Newly emerged adults were then moved into metal net cages (2 × 1.5 × 1.5 m) and fed for about two weeks with fresh twigs of maple (*Acer saccharinum* L.) to allow adult sexual maturation and mating. Fresh maple logs (10 cm in diameter and 30 cm long) were then placed in the same cages for egg laying in the phloem.

CLB. Several adults of CLB were collected manually from an urban park in Milan (North Italy) in July 2013 (45°29'N 9°05'E), placed in plastic cages (30 × 20 × 18 cm) and fed with fresh maple twigs. After a few days, small maple twigs (2-3 cm diameter and 10-15 cm long) were added to the cages to assure egg laying under the bark of the host tree.

YLB. A laboratory rearing of YLB was set up in summer 2013 from adult beetles emerged from infested logs of fig trees sampled in Anzano del Parco (45°49'N 9°13'E) in North Italy. Four to ten pairs of newly emerged YLB adults were placed in plastic cages (30 × 20 × 18 cm). Three times a week the adult beetles were provided with new fresh fig twigs (1.5-2.5 cm diameter and 10-15 cm long) in which to lay eggs.

The adults of the three reared species could move freely in the rearing cages kept in climatic chambers (temperature 25 ± 0.5 °C, photoperiod L16:D8, relative humidity 65 ± 0.5%) in order to mate repetitively and ensure random oviposition in twigs and logs of the host trees (maple and fig). Infested logs were checked once a week and the freshly laid eggs collected one-by-one by stripping the bark with a cutter. The eggs were then moved singly into the diet. In order to avoid any parental effect, eggs were randomly collected from at least ten twigs and logs.

Diet composition and preparation

The tested artificial diet, modified following Cappellozza *et al.* (2005) and provided by the Honey Bee and Silkworm Research Unit of the Italian National Council for Agricultural Research and Economics (table 1), was used for the longhorn beetle rearing according to the

Table 1. Composition of the tested diet, modified from Cappellozza *et al.* (2005).

Ingredients	g
Mulberry leaves powder	40
Soybean meal	30
Wheat meal	10
Corn starch	5
Citric acid	4
Ascorbic acid	2
Wesson's salt mix	3
Agar	4.2
Vitamin mix	0.399
Sorbic acid	0.200
Propionic acid	0.691
Chloramphenicol	0.010
Beta-sitosterol	0.500
Total	100

protocol preliminarily tested on YLB (Lupi *et al.*, 2015b). The dry powder was hydrated (2.6 g of distilled water per g of dry diet), cooked in microwave oven (4 min. at 600 W), and then cooled at room temperature. The diet resulted in an easy-to-handle cream poured into 60 and 90 mm diameter Petri dishes, according to larval size, and stored in a refrigerator (4 °C) until use. Approximately 3 g and 5 g of dry diet powder were required to fill 60 mm and 90 mm Petri dishes, respectively.

Insect rearing

One hundred eggs per species, extracted from infested twigs and logs, were moved singly to a Petri dish and placed in a small chamber dug in the diet previously warmed to room temperature. Petri dishes were kept in climatic chambers at constant conditions (temperature 25 ± 0.5 °C, photoperiod 16L:8D and relative humidity 60 ± 0.5%). The larvae born in the Petri dishes were checked weekly and moved to a new Petri dish with fresh diet. Larval mortality, larval weight and head capsule width were recorded weekly. Head capsules were also collected after each larval moulting, and their width measured.

According to Keena (2005) and Keena and Moore (2010), 12 weeks after hatching ALB and CLB larvae were exposed to a 12 weeks chilling period (10 ± 1 °C temperature and 12L:12D photoperiod) in order to stimulate pupation. After chilling, larvae were moved again to rearing conditions (25 ± 1 °C temperature and 16L:8D photoperiod) in order to complete development and pupate. ALB and CLB larvae not pupating within 36 weeks from egg hatching were exposed to a second chilling period of 12 weeks. Instead, according to Shintani *et al.* (1996) and Lupi *et al.* (2013), YLB larvae did not require a chilling period before pupation.

Until the third larval instar 60 mm Petri dishes were used, while larger larvae were moved to 90 mm Petri dishes. Lastly, before pupation mature larvae were moved to plastic jars 95 mm in diameter and 40 mm in height, giving the space required for larval pupation and adult emergence.

After emergence, each adult was held for two days in the plastic jar to allow full cuticle sclerotization. Adults were then sexed according to Lingafelter and Hoebeke (2002), placed singly in plastic cages (33 × 35 × 45 cm) and fed with fresh maple (ALB and CLB) or fig twigs (YLB). Adult longevity was then recorded daily.

Data collection lasted until the last larvae pupated and adult emerged or died.

Insect measurements

The width of the larval head capsule was measured weekly on live larvae using an optical stereoscope (Wild Heerbrugg M5A, Leica Geosystems GmbH, Heerbrugg, Switzerland) provided with graduated oculars. The larval instar was assessed according to the widths of the larval head capsules and the exuviae found after the larval moults.

Body length from the head to the elytra apex was measured with a calliper in all adults emerged from the artificial diet. In summer 2013 and 2014 wild adult beetles (40 males and 40 females) of each investigated species were collected in the field from the same infestation areas, i.e. the same populations reared in lab conditions, and kept in ethyl alcohol 70% for comparative measurements with adults emerged from diet.

Rearing cost per adult beetle

The amount of diet required by each larva in the research (L_n) was calculated taking into account the number of development weeks (w.), omitting chilling and pupation periods (if occurred) when larvae do not feed. Approximately 5 g of dry diet powder per week were required by each larva, *i.e.* the amount of diet needed to fill a 90 mm Petri dish. The total amount of dry diet required for the development of each larva was thus:

$L_n = 5\ g \times (\text{total w.} - \text{chilling w.} - \text{pupation w.})$ [1]

Nevertheless, not all larvae completed their development as some died before pupation, therefore affecting the total rearing cost.

Considering that the dry diet costs about 27 US$/Kg, the final cost (mean cost per adult) for obtaining an adult was thus:

$(\sum L_n \times 0.027\ \text{US\$/g}) / (\text{no. emerged adults})$ [2]

The cost of reusable containers and Petri dishes was considered as negligible. Work hours and supplies costs depend on many factors such as amount of beetle rearing, laboratory facilities and staff experience. Their influence on the total cost is variable and for the purpose of this study, only the diet cost itself has been reported.

Statistical analysis

To assess the rearing performance on the diet, development time and adult longevity were compared between species by one-way analysis of variance (Anova), while adult size was compared between wild and reared individuals. Larval weight and larval head capsule size were analyzed by a repeated measures analysis of variance (RMAnova).

Homogeneity of variance was tested by Cochran's test (test C), and normality by Kolmogorov-Smirnow's test (test D); when necessary, data were log [X' = log(x + 1)] or arcsin ($X' = \arcsin\sqrt{x}$) transformed to obtain homo-

geneous data and normal variance. Wherever significant differences occurred, Tukey's Honest Significant Difference (HSD) multiple comparison test was applied for mean separation (Zar, 1999). Differences at a 0.05 level of confidence were considered significant. Statistical analyses were performed using the R-project software (R Core Team 2015). Values are reported as mean ± standard error (SEM).

Results

Larval survival and adult production

All three tested species were able to fully develop until the adult stage on the diet (figure 1). ALB had the lowest hatching rate (60%), YLB the highest (81%), while CLB showed an intermediate value (77%). With respect to the number of hatched eggs, larval survival and adult production was 74.1%, 24.7% and 23.4% for YLB, CLB and ALB, respectively, with mortality occurring mainly during the first two weeks of the rearing with mean values of 22%, 11% and 36.7%, respectively. After this initial period, mortality strongly decreased, although continuing slightly until the first chilling period of ALB and CLB. No mortality was recorded during pupation for any species.

Development time

Development duration varied according to species and instar (table 2). YLB showed the shortest larval (14.10 ± 0.55 weeks) and pupation (2 ± 0.08 weeks) development, ALB and CLB the longest (33.23 ± 0.71 and 30.50 ± 0.62 weeks for larvae exposed to one chilling period, and 3.07 ± 0.20 and 2.88 ± 0.08 weeks for pupae, respectively) (Anova, $F = 214.9$, $df = 2; 93$, $P < 0.01$; Tukey test, $P < 0.05$, table 2).

Although some CLB (10.5%) and most ALB (92.8%) larvae pupated after the first chilling period, the remaining ones (89.5% CLB and 7.2% ALB) required a second chill treatment. Of these, 11% of CLB larvae died during the second chill, 41% did not pupate and continued moulting until death, and only 48% pupated producing adults. The need for a second chilling period by some ALB and CLB larvae hence made the mean development time of these two species (61.59 ± 2.26 and 37.29 ± 1.09 weeks for CLB and ALB, respectively) substantially longer than in YLB (16.1 ± 0.57 weeks) (Anova, $F = 255.1$, $df = 2; 93$, $P < 0.001$) (table 2). However, some individuals in all species required a longer development time than the majority, with a maximum of 22, 50 and 78 weeks being recorded for YLB, ALB and CLB, respectively.

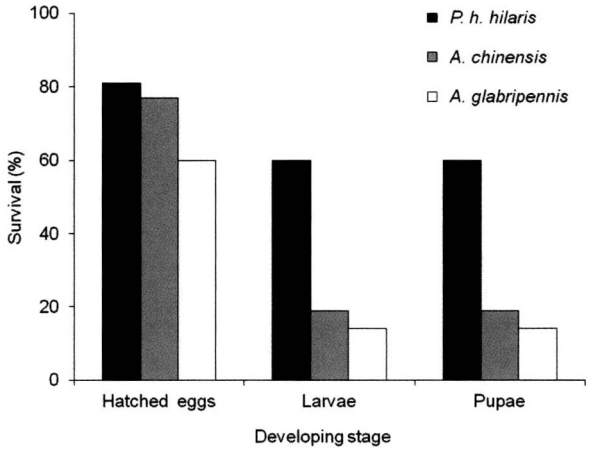

Figure 1. Survival (%) of each developing instar of *P. h. hilaris*, *A. chinensis* and *A. glabripennis* reared on the artificial diet.

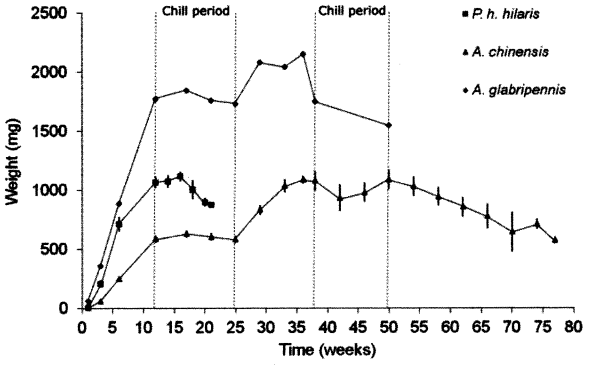

Figure 2. Larval weight (mean ± SEM) of *P. h. hilaris*, *A. chinensis* and *A. glabripennis* over time (weeks).

Larval weight

According to larval development, weight showed common patterns in all three reared species (figure 2), with a rapid increase during the weeks before chilling, a slight decrease during chilling, rising again afterwards and reducing a little in the last 2 weeks before pupation. During the last weeks CLB showed a constant and progressive weight loss due to the continuous moulting of non-pupating larvae (figure 2). In all species, the increase in larval weight was very quick in the first three weeks. The weight varies according to species through time (RMAnova, $F = 3.25$, $df = 57$, $P < 0.01$).

Head capsule size and larval instars

The number of larval instars was determined by the progressive measurement of the head capsules. The moults required for pupation varied among and within

Table 2. Duration (in weeks ± SEM) of larval and pupal development of *P. h. hilaris*, *A. chinensis* and *A. glabripennis* reared on the artificial diet. Different letters mean significant differences among species (Anova test, $P < 0.05$).

Species	Larvae (requiring one chill)	Larvae (requiring two chills)	Pupae	Mean duration (egg-adult)
P. h. hilaris	14.10 ± 0.55 b	-	2.00 ± 0.08 b	16.10 ± 0.57 c
A. chinensis	30.50 ± 0.62 a	60.50 ± 1.39 b	2.88 ± 0.08 a	61.59 ± 2.26 a
A. glabripennis	33.23 ± 0.71 a	47 a	3.07 ± 0.20 a	37.29 ± 1.09 b

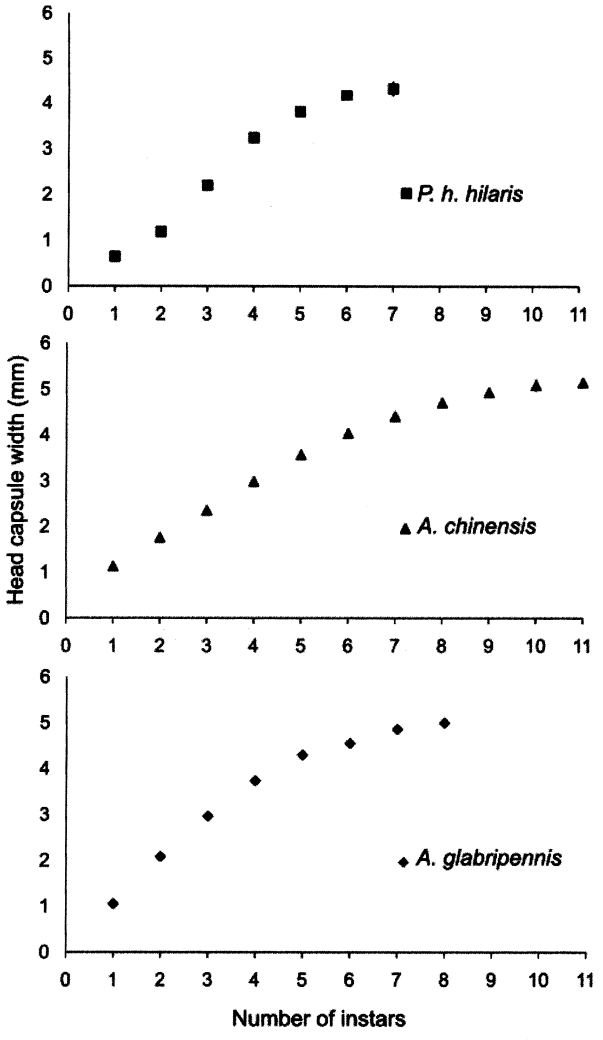

Figure 3. Maximum number of larval instars of *P. h. hilaris*, *A. chinensis* and *A. glabripennis* reared on the artificial diet according to mean larval head capsule width (mm).

species; YLB showed from 5 to 7 larval instars, with the majority of larvae reaching pupation after 5 (51.7%) or 6 (41.3%) instars. CLB needed from 6 to 11 moults, although all larvae requiring just one chilling period pupated at instar 6, while most individuals exposed to a second chilling pupated at instar 9 (35.7%) or 10 (35.7%). Lastly, ALB had larvae pupating at instar 7 (43%) and 8 (57%) (figure 3). The head capsule size differs significantly depending by species and time (RMAnova, $F = 34.16$, $df = 57$, $P < 0.01$).

Adult size, sex-ratio and longevity

YLB adults emerged from larvae reared on the artificial diet resulted significantly bigger than wild specimens, both for males (26 ± 0.8 versus 21.3 ± 0.9 mm, respectively) (Anova, $F = 7.22$, $df = 1; 58$, $P < 0.01$) and females (25.4 ± 0.4 versus 22.5 ± 0.3 mm, respectively) (Anova, $F = 24.58$, $df = 1; 82$, $P < 0.001$) (table 3). ALB adults from rearing were also bigger than wild ones, although only the females showed significant differences (31.3 ± 0.6 and 25.8 ± 0.4 mm, respectively) (Anova, $F = 25.88$, $df = 1; 48$, $P < 0.001$), while males did not (25.2 ± 0.4 and 22.5 ± 0.8 mm, respectively) (Anova, $F = 3.12$, $df = 1; 46$, $P = 0.09$). Unlike the other two species, CLB adults reared on the artificial diet were smaller than wild specimens (table 3), resulting in significant differences both for females (27.1 ± 0.3 and 29.9 ± 0.51 mm) (Anova, $F = 24.58$, $df = 1; 52$, $P < 0.001$) and males (24.0 ± 0.7 and 28.3 ± 0.5 mm) (Anova, $F = 7.22$, $df = 1; 47$, $P < 0.01$) (table 3).

In all the reared species more females emerged than males. More specifically, 30% males and 70% females in YLB, 36.8% males and 63.2% females in CLB, and 42.9% males and 57.1% females in ALB. The observed sex ratios, reported as males over females, were hence 0.42, 0.58 and 0.75 for YLB, CLB and ALB, respectively.

The lifespan of emerged adults differed significantly between species (Anova, $F = 5.46$, $df = 2; 93$, $P < 0.01$). ALB adults survived shorter (62.9 ± 9.14 days) than YLB (119.3 ± 12.24 days), while CLB adults had median longevity (102.8 ± 17.91 days). Lastly, males and females survived equally in each species (Anova, $F = 0.497$, $df = 1; 93$, $P = 0.485$).

Rearing cost per adult

According to equation [1], to fully develop each larva medially required 70.5, 111 and 166 g of dry diet for YLB, ALB and CLB, respectively. The mean cost of diet required for each emerged adult estimated according to equation [2], and considering the different rearing performance of each species, is hence 2.0, 8.1 and 16.1 US$ for YLB, ALB and CLB.

Discussion and conclusions

The tested diet allows insect development from egg to adult in all three species YLB, CLB and ALB. The perfectly developed adults are similar in size and morphology to field collected individuals, able to feed and sur-

Table 3. Mean (± SEM) size (mm) of adults of *P. h. hilaris*, *A. chinensis* and *A. glabripennis*, reared on the artificial diet compared to wild specimens. Asterisks mean significant differences among reared and wild adults (Anova test).

Species	Females		Males	
	From diet	Wild	From diet	Wild
P. h. hilaris	25.4 ± 0.4 **	22.5 ± 0.3	26.0 ± 0.8 *	21.3 ± 0.9
A. chinensis	27.1 ± 0.3 *	29.9 ± 0.51	24.0 ± 0.7 **	28.3 ± 0.5
A. glabripennis	31.3 ± 0.6 *	25.8 ± 0.4	25.2 ± 0.4	22.5 ± 0.8

* $P < 0.05$; ** $P < 0.01$.

vive for several months after emergence. The diet used in this study therefore satisfied Cohen's (2015) first assumption as it simulated host taste, odour and texture: larvae accepted the diet, started to feed on it and entered the food matrix as they would have done in wood. Even though adult fecundity was not here reported, preliminary investigations demonstrated that adults emerged from the diet were fertile and produced fertile eggs, with fecundity similar to that recorded in wild adults (Lupi *et al.*, 2015b).

Our results concerning YLB rearing, in which 74% of hatched eggs produced new adults, are in line with those observed in Japan using similar diets (Shintani *et al.*, 1996; 2003). Rearing performance of ALB and CLB was instead poorer but very similar to each other (23.3% and 24.7%, respectively), and mainly due to the high egg and young larva mortality. Nevertheless, Faccoli and Favaro (2016) demonstrated how mortality of ALB eggs and first-instar larvae may be extremely high also in field conditions regardless to the host-trees. As ALB and CLB are species highly polyphagous on broad-leaves, including mulberries (Hérard *et al.*, 2009; Sjöman *et al.*, 2014), we expected that they would easily adapt also to a mulberry-based diet.

The use of host tissues (leaves) different from those usually exploited by the insects (phloem and xylem) not affected the general insect development. In studies carried out by Scrivener *et al.* (1997) the larvae of *P. h. hilaris* digested the major components of mulberry leaves. Besides, the artificial diet suggested by Dubois *et al.* (2002) for ALB rearing accounted only for 20% of total weight in phloem-cambium of *A. saccharum* and produced about 41% adults; Keena (2005) did not use host plant tissues in her diet, but however obtained a relevant number of viable ALB adults (63.5%). Our low percentage of emerging ALB and CLB adults may be due to different reasons. ALB showed a very high mortality of young larvae mainly within the first 3 weeks after hatching, due to inability of the newly born larvae to enter the diet and start feeding. During a preliminary trial, two weeks old larvae were collected directly from the bark and moved to the diet, showing a much higher survival rate than larvae born in the diet (unpublished data). As suggested by Murakoshi (1981), disinfection of eggs may reduce mortality of newly born larvae. CLB instead had higher survival of newly born larvae, but its mortality continued constantly over time. The longer CLB development has also negatively affected its larval survival: larvae moulted repetitively avoiding pupation and finally dying. In this respect, because little is known about the effects of temperature on CLB, we applied the chilling protocol proposed by Keena (2005) for ALB, although some differences may occur for CLB. The exposure of CLB larvae to a chilling period should hence be more deeply evaluated to increase pupation rate, and reduce both development time and larval mortality.

P. h. hilaris was able to develop in the diet more rapidly and successfully, and without needing a chilling period. The mean development time required by YLB (only about 16 weeks) suggests the possibility of having two generations per year, as reported in Japan (Shintani *et al.*, 1996). Instead, ALB needed a longer period (about 37 weeks) for the complete egg-adult development and most larvae pupated only after one chilling period 12 weeks long (only 7.2% of individuals required a second chilling period). This suggests the rearing of mostly univoltine populations. Lastly, the very long mean development time of CLB was affected by the large part of the population requiring a second chilling period (table 2), producing uni- or semivoltine cohorts of insects.

All larvae reared on the diet showed a great weight increase during the first weeks of their development. An extraordinary growth (up to 5,000% of the weight of the newly hatched larvae) was observed during the first three weeks of YLB development. The growth trend was similar in all the tested species, although lower in ALB and CLB. A strong weight increase was observed during feeding periods, a slight decrease during chilling periods (for the species exposed to this treatment), and again a slight weight decrease near pupation. Longhorn beetles usually have a variable number of larval instars in relation to environmental conditions occurring during development (Shintani *et al.*, 1996; Lingafelter and Hoebke, 2002). For instance, YLB larvae may have supernumerary moults when exposed to unsuitable development conditions, while diapause requirement is under photoperiod control (Shintani *et al.*, 1996). In this respect, our results concerning YLB and ALB larval instars are in line with previous observations (Shintani *et al.*, 1996; Keena, 2005). The results for CLB are also consistent with the literature, as Lingafelter and Hoebeke (2002) report 7-9 larval instars for univoltine individuals (exposed to one chilling period) and 11-15 instars for semivoltine ones (exposed to two chilling periods).

According to the literature, YLB is the smallest species, whereas both the *Anoplophora* species are larger and similar in size (Lingafelter and Hoebeke, 2002; Fukaya, 2004; Lupi *et al.*, 2015a). YLB females were the smallest adults obtained from the diet, while CLB showed the smallest males, although all recorded values fell within the size range known for these species. YLB and ALB adults of both sexes emerged from the diet were significantly bigger than wild adults. Because fecundity, breeding performance and longevity of longhorn beetles are strictly related to their body size (Hanks *et al.*, 1996; 2005; Fukaya, 2004), our results suggest that, at least for these two species, the tested artificial diet and the applied rearing protocol provide a breeding performance quantitatively and qualitatively comparable with, or even better than, host-tissues and wild populations. The smaller size of CLB adults from the diet with respect to wild adults was partially compensated by their high longevity (102.8 days) and fertility, as the lifespan is longer and more eggs are laid (Smith *et al.*, 2002).

The number of emerging fertile females is another crucial point in laboratory rearing. In order to increase egg production, a number of females greater than males would be required, given that a few males can mate with

many females (polygyny). Although in the literature it is usually reported to be equally distributed (Adachi, 1994), during the present trials the sex-ratio of emerging adults was probably somehow influenced by diet composition or rearing environment conditions, leading to a higher proportion of females (70% and 63% of total individuals for YLB and CLB respectively), a favourable result for sustainable laboratory rearing. ALB sex-ratio was instead close to 1 (57% of females), confirming previous studies conducted on the same population in the field (Faccoli *et al.*, 2015).

Costs for ALB, CLB or YLB rearing are scarce in the literature. The only available data are reported by Keena (2005), referring to a mean rearing cost of about 21 US$ per ALB adult, not including start-up and overhead costs (totalling 10 US$), but accounting general costs such as salaries and equipment maintenance. The diets proposed by Dubois *et al.* (2002), according to their compositions, were even more expensive. The calculations of the cost per beetle for the three species reared in the present study are not directly comparable to previously published information for ALB, since handling costs and other general costs were not included in our calculation. However, despite a lower rearing performance, i.e. lower percentage of emerging adults, the diet tested in our experiment allowed the cost to be reduced to only 8.1 US$ per ALB beetle, making it extremely cheap, and even cheaper for YLB (about 2 US$ per adult).

Further evaluations of the effects of diet composition and rearing conditions (mainly temperature) on development of YLB, ALB, CLB and other invasive exotic longhorn beetles are needed to improve the general rearing performance of these pests on an artificial diet. The evaluation of fecundity and fertility of progeny produced by adults reared on this diet should be also evaluated, attempting to rear a second generation on the same diet. These aspects may be of crucial importance for future studies, as in some case there are more severe impacts of a poor diet in the second generation through maternal effects. The effects of different chilling temperatures and chilling periods on different longhorn beetle species still need to be carefully evaluated because the shorter the chilling period is, the shorter is the developmental time, reducing rearing costs and increasing the mean number of generations per year. The easy, continuous and cheap production of a large amount of adults in standard conditions should be extremely useful for studies focused on the biology and ecology of these species. Moreover, our results are not just useful for biological and ecological studies carried out in standard conditions but also for studies looking at the efficacy of chemical treatments for quarantine purposes (e.g. fumigants or pesticides), which normally require huge numbers of insects. In conclusion, a better understanding of the mechanisms governing larval growth and pupation under different rearing conditions is crucial to help in predicting the timing of the various insect instars and thus support decisions about eradication and control treatments (Keena, 2005).

Acknowledgements

The research was supported by the project "Insects and globalization: sustainable control of exotic species in agro-forestry ecosystems (GEISCA, PRIN 2010-2011)", financed by the Italian Ministry for Education, University and Research (Project leader: *Alma Mater Studiorum* University of Bologna), and partially supported by the University of Padua (ex-60% projects). A special thanks to Alison Garside for the linguistic proof-reading of the manuscript.

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Received May 5, 2016. Accepted January 26, 2017.