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Biology of *Euwallacea interjectus*, an emerging poplar pest, reared on an ambrosia beetle artificial diet and medium of fungal symbiont

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Abstract

Euwallacea interjectus, a recently discovered pest in poplar plantations, poses a significant economic threat due to its role in causing widespread tree mortality. This pest's cryptic behaviour has hindered research and control efforts, making laboratory rearing a valuable tool for studying its development and biology. We investigated the development period and biological characteristics of *E. interjectus* using artificial diets and fungal medium. Our findings revealed that the development time for eggs, larvae, and pupae averages approximately 6, 18, and 6 days, respectively. Notably, first and second instar larvae displayed peak moulting periods at 3.45 ± 0.64 SD and 7.92 ± 1.77 SD days, respectively. Furthermore, we measured head capsule widths of postmolt larvae, yielding values of 318.02 ± 7.38 SD μm for first-instar larvae, 403.01 ± 11.08 SD μm for second-instar larvae, and 549.54 ± 20.74 SD μm for third-instar larvae. Our research also uncovered a positive correlation between the number of progeny (eggs, larvae, pupae, and adults) and the mean length of the gallery system. Interestingly, the haplodiploid reproductive strategy did not significantly affect the number of offspring produced by the foundress. Additionally, we observed that foundresses displayed higher fecundity when subjected to nutrient-rich diets as compared to nutrient-poor diets. Our results will deepen our understanding of the biology of *E. interjectus* and provide criteria for larval instar classification. Additionally, managing nutrient availability within the colony could be considered a viable approach to regulating population size.

Introduction

Euwallacea interjectus Blandford (Coleoptera, Curculionidae: Scolytinae) is the ambrosia beetle species that can invade trees and excavate the gallery. These beetles establish a symbiotic relationship with fungi providing nutrition for development (Batra, 1963; Biedermann *et al.*, 2013). Symbiotic fungi are stored in specialised transporting organs called mycangia in the ambrosia beetle (Hulcr and Cognato, 2010; Hulcr and Stelinski, 2017; Skelton *et al.*, 2019). After invading the tree, the beetles release the fungus from mycangia into the gallery (Batra, 1963). The fungal-beetles help them adapt to different hosts and environments during long-term evolution (Hulcr and Skelton, 2023).

E. interjectus has a wide host range, and related invasion has been reported in Japan, Argentina, China, and the United States. The beetle not only mass invades water-stressed poplars and boxelder maples (Acer negundo L.) (Aoki *et al.*, 2019; Landi *et al.*, 2019), but healthy trees are also attacked under the right conditions (Knížek and Beaver, 2007). The rare characteristic of *E. interjectus* that colonise healthy trees is making them a new ecological and economic threat (Kajii *et al.*, 2013; Wang *et al.*, 2021). In addition to devastating the appearance and monetary value of vegetation like other ambrosia beetles, *E. interjectus* can act as a vector for the pathogen (*Ceratocystis ficicola*) that causes fig wilt disease (Kajii *et al.*, 2013; Jiang *et al.*, 2022). In Japan, an infestation of *E. interjectus* has dealt a severe economic blow to fig orchards (Morita *et al.*, 2012). In China, poplar trees (*Populus* × *canadensis* Moench) invaded by *E. interjectus* also showed wilting and serious decline (Wang *et al.*, 2021; Lai *et al.*, 2022). The Poplar tree, widely planted in various provinces of China, is an essential economic plant. Therefore, understanding the biology of *E. interjectus* can help to develop rational control measures to prevent further invasion.

Laboratory rearing can help us understand the ambrosia beetle's developmental cycle and behaviour to develop control strategies. Saunders and Knoke (1967) originally explored an artificial diet supplemented with cacao sawdust and successfully reared *Xyleborus ferrtgineus*. Considering the physical properties of sawdust provide a tree-like structure and living environment, artificial diets supplemented with sawdust have been more widely adopted in rearing studies of ambrosia beetles. A variety of the ambrosia beetle has been successfully reared and

studied on their biological characteristics, such as *Xyleborus pfeili*, *Euwallacea fornicatus*, *Xyleborus bispinatus*, *Xyleborus volvulus* (Mizuno and Kajimura, 2002, 2009; Cooperband *et al.*, 2016; Menocal *et al.*, 2017; Cruz *et al.*, 2018). In addition, Freeman *et al.* (2012) successfully raised *Euwallacea aff. fornicata* Eichhoff using fungal petri dishes. They reported that larvae can obtain nutrients by eating fungi on the fungal culture medium (PDA). The open-air environment of the fungal medium may be suitable for detecting the development of larvae.

In the past decade, *E. interjectus* has caused not only severe economic damage to native tree species in its invaded regions but also its native Asian range (Lai *et al.*, 2022). Currently, knowledge of *E. interjectus* is limited to its symbiotic fungus and some reports of invasiveness, with little biological research reported. However, understanding the biology of *E. interjectus* is vital for their monitoring and control. In the native environment, the cryptic lifestyle of the beetle may be a challenge for the study of biology. The artificial diet and fungal petri dish rearing offer assays for exploring the biology of *E. interjectus* under the lab. Indeed, there have been many successful experiences of laboratory rearing to draw on, such as *X. pfeili*, *Xyleborus saxesenii*, etc. (Mizuno and Kajimura, 2002; Biedermann, 2010). However, there are still exciting challenges in the laboratory rearing ambrosia beetles. For example, (i) the developmental time of the second and third instar larvae has not been determined separately; (ii) the instars of larvae are more judged by experience rather than a specific indicator.

In this study, we aimed to explore a suitable rearing technique for *E. interjectus*, which can facilitate obtaining mass beetles in the laboratory. In addition, the other goal is to elucidate the biological characteristics of laboratory-rearing beetles. The specific contents were to (1) evaluate rearing survival and fecundity; (2) clarify the life cycle and developmental stages; (3) determine the process of gallery construction; (4) investigate the biological characteristics (dispersal behaviour, haplodiploid reproduction) during the developmental stages.

Materials and methods

In the studies on the biology of *E. interjectus*, artificial diet and fungal medium rearing were used, respectively. The studies on rearing success, fecundity, developmental stages, gallery construction, dispersal behaviour, haplodiploid reproduction, and sex ratio used the artificial diet. In addition, fungal medium rearing was used for the studies on egg incubation, larval instars, and larval pupation.

Rearing with artificial diet

Insects

Two sources of *E. interjectus*: (1) F0 generation; Female adults were collected through the dissection of infested logs extracted from host trees. The logs used in the study were obtained from Dongtai, Jiangsu (120°49′10″E, 32°52′08″N). (2) F1 generation: Female adults dispersing from the artificial diets introduced the F0 generation.

Treatment and introduction: To prevent bacterial and non-mycangia fungal contamination, beetle surfaces were sterilised with 75% ethanol for 5 s and dried for 5 s on a piece of filter paper. An initial hole (2 mm deep) was poked into the diet surface of each tube to facilitate boring activity, and a single female adult was introduced into the diet.

Table 1. Three different artificial diets were tested in the initial rearing

Ingredients	Diet-1	Diet-2	Diet-3
Wesson's salt mixture	0.5 g	–	0.5 g
Yeast	5 g	–	5 g
Casein	5 g	–	5 g
Starch	5 g	–	5 g
Sucrose	10 g	–	10 g
Agar	20 g	20 g	20 g
Streptomycin	0.35 g	–	0.35 g
Wheat germ oil	2.5 ml	–	2.5 ml
100% of ethanol	5 ml	–	5 ml
Deionised water	500 ml	500 ml	500 ml
Poplar sawdust	100 g	100 g	–

Except for this trial, the rest of the rearing used Diet-1.

Artificial diet

In the initial stages of exploring the diet composition, three different diets were developed based on the method described by Maner *et al.* (2013) (table 1). The steps for making sawdusts and diets are shown in the supplementary diet. The main diet (Diet-1) used in rearing was made of sawdust and rich nutrients. To explore the effect of ingredients on female oviposition and offspring development, two additional diets were prepared. The second diet (Diet-2) only consisted of sawdust, agar, and deionised water. The third diet (Diet-3) eliminates sawdust compared with Diet-1.

Developmental stages and gallery construction

After F0 and F1 females were introduced to the diet, at least four tubes of the two generations were dissected at intervals for two days. Before dissecting, offspring above the diet surface were removed and counted. Then, the diet material was removed from the tube, and the galleries were exposed using tweezers. All development stages of progeny, including eggs, larvae, pupae, and adults, were collected and quantified.

According to the theory of Mizuno and Kajimura (2002), the gallery system consists of a main gallery (M), branch tunnels (B), and side tunnels (S). The length of branches was measured for each dissection to explore the change in the gallery system with the development of the offspring. Measuring the length is done by placing the string along the gallery.

Sex ratio

Previous studies have used methods of dissecting diets or collecting dispersed adults to study the sex ratio of the ambrosia beetle (Biedermann, 2010; Cooperband *et al.*, 2016). The dispersal behaviour and sex ratios of *E. interjectus* on fig trees have been reported (Jiang *et al.*, 2021), but no relevant experiments have been noted under laboratory rearing. To judge whether the sex ratio conforms to the concept of LMC, two methods of determining the sex ratio were verified. (1) After female adults were introduced to the diet, observations were conducted throughout the period of adult dispersion. Examined the surface of the diet daily, and the dispersing adults were transferred to fresh Petri dishes to prevent confusing with the adult of next day. The number of dispersed adults on the surface was quantified

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daily for each tube. (2) Also, sex ratios of F0 and F1 were determined by collecting adults and pupae during dissection. Only colonies dissected between 33 and 42 days were used for sex ratio determination.

Haplodiploid reproduction

During the long-term evolution process, the ambrosia beetle formed typical characteristics of haplodiploid reproduction, in which fertilised females produce female eggs and unfertilised females produce male eggs (Peer and Taborsky, 2005). To determine the fecundity of fertilised and unfertilised females, 1 adult (♀) and 2 adults (♀♂) were introduced to the diet separately. Unmated adults are obtained by rearing male and female pupae separately. After the pupae emerge, the newly emerged male and female adults are introduced into the fungal medium to obtain symbiotic fungi. After the body colour of the virgin females turned black (about 7 days), each adult was sterilised on the body surface and introduced into the diet. These diets were dissected during 20–30 days.

In addition, it is unknown whether unfertilised females can produce female offspring, how many and when they have female offspring. To figure it out, 31 virgin female adults were introduced to the artificial diet. 16 colonies were dissected between 30–40 days, and others were dissected between 50–60 days.

Fungal medium rearing

Creating a bare living space that provides nourishment and observation is essential. The fungal medium may meet this demanding requirement. The preparation of fungal medium is shown in the supplementary diet. Fungus medium are used for the rearing of eggs and larvae. All eggs were collected from artificial diets (Diet-1) introduced into F0 generation adults (the method of obtaining F0 is the same as above). The purpose of fungal medium rearing is to determine the developmental period of each stage and their survival. This study defined egg survival (in %) as the percentage of eggs that successfully incubated. Larvae survival (in %) is defined as the percentage of larvae that successfully pupated. Pupae survival (in %) is measured as the number of eclosion pupae.

Eggs

To determine the developmental timing of the eggs, the diet on 3rd day was dissected to ensure that all the eggs were fresh (Females began laying eggs on the third day). All collected eggs ($N = 31$) were reared on paper placing in the fungal medium. Observe whether the eggs hatch into first-instar larvae every day under a stereoscope, and record the number and time of egg incubation.

Larvae

All larvae are guaranteed to be first instar larvae just hatched from eggs as follows. Eggs are obtained and reared in the same way as in the previous section. Observe the eggs three times a day under a stereoscope, place the hatched first-instar larvae in the fungal medium, and control the number of larvae in each fungal medium to about 30.

In the trial, 219 first-instar larvae were reared in fungal medium at 25°C. The larvae were observed under a microscope twice a day, and the number of dead larvae and moulting were recorded. Plates for rearing larvae were replaced every day to maintain a clean living environment. All pupated larvae will be used in the next phase of the experiment.

In order to determine the changes in head capsule width of larvae during growth, a batch of larvae were reared individually, and the larvae after moulting were taken from each instar and the head capsule width was measured. Take 40 moulting larvae from each instar. In order to ensure the accuracy of the larval head shell width and facilitate measurement, all collected moulted larvae were soaked in paraformaldehyd fixative (neutral) (Servicebio, G1101-500ML).

Pupae

All pupae were reared separately by sex in Well plate (12 wells) containing moist cotton cloth. Pupae on the same day should be placed in the same well for observation and statistics. Observe and record the number of dead pupae every day. Dead pupae should be cleaned up in time to avoid infection.

Statistical analysis

All data in this study were analysed using IBM SPSS Statistics 27.0 and Origin 2022. A linear regression model was used to predict the relationship between the number of offspring and the length of the gallery system (SPSS). Fitting curves were used to indicate the peak period of adult dispersal and peak moult period of first and second instar larvae (Origin). The chi-square test was used to analyse the difference in the breeding success (SPSS). For analyses comparing two sets of nonparametric data, use the Mann–Whitney U test to compare the data sets (SPSS).

Results

Breeding success

The situation of colonies was classified in three styles: (1) no offspring in the gallery, (2) dead offspring in the gallery, (3) live offspring in the gallery (fig. 1C). Rearing success was defined as the presence of live offspring in the diet, otherwise considered failures. Regardless of the presence (Diet-1) or absence (Diet-3) of sawdust in the diet, female beetles excavated galleries and oviposition. However, female beetles had higher breeding success in artificial diets containing poplar sawdust (Diet-1) ($\chi^2 = 23.350$, $df = 1$, $P < 0.001$). In terms of fecundity, foundress (F1) showed greater fecundity in Diet-1 supplemented with various nutrients than in Diet-2 (Mann–Whitney U test: $Z = -3.373$, $P < 0.001$, $N = 25 + 78$).

In diet-1, the gallery productivity of F0 and F1 between 33–41 days was 25.7 ± 18.7 SD and 21.0 ± 13.8 SD, respectively. There was no significant difference in the gallery fecundity between the F0 and F1 generations at 33–41 days (Mann–Whitney U test: $Z = -0.775$, $P = 0.438$, $N = 61 + 41$). Gallery productivity was defined as the number of eggs, larvae, pupae, and adults. Although the definition of gallery productivity differs from Manner's and Biedermann's, the study's results are similar to those of predecessors. In addition, there was also no significant difference in breeding success between the F0 and F1 generations with diet-1.

Developmental stages

The life cycle of *E. interjectus* consists of 4 stages, including eggs, larvae, pupae, and adults (fig. 2). Although there was variation in

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Figure 1. Records in the process of rearing and dissection. (a) The artificial diet covered with symbiotic fungi. (b) A large number of long cylindrical sawdust can be observed on the surface. (c) Eggs, larvae, pupae, and adults are randomly distributed in the same gallery. (d) The main gallery and the branch tunnel within the diet.

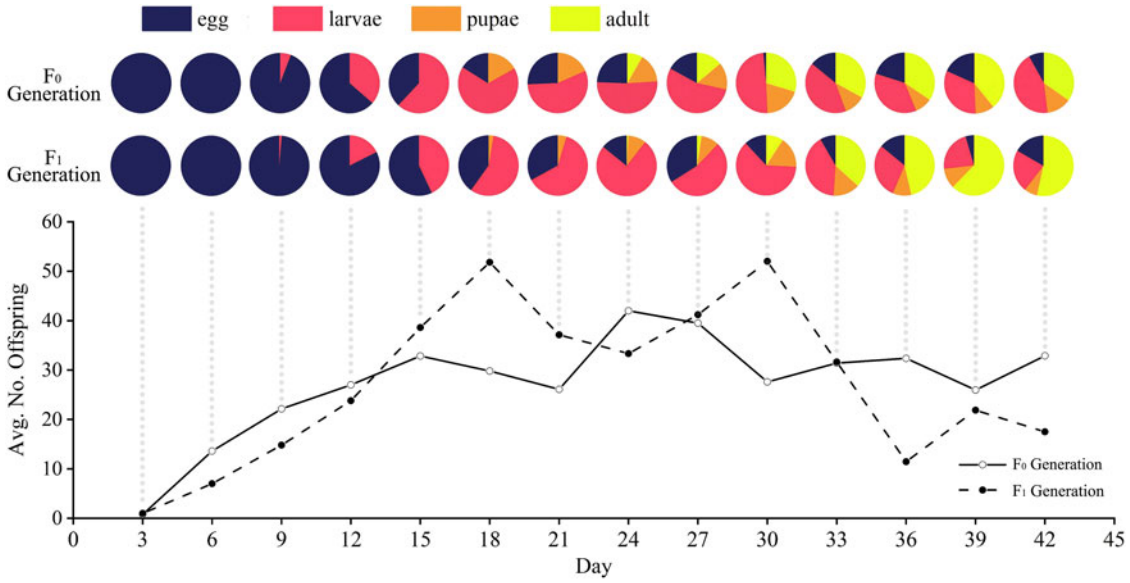

Figure 2. The average number of offspring and the proportion of developmental stages in the F0 and F1 generations. The detailed percentages are shown in table 2.

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Table 2. The Number of offspring in each stage of the F0 and F1 generations at different times

Generation	Per cent%	Time (DA)												
		3D	6D	9D	12D	15D	18D	21D	24D	27D	30D	33D	36D	39D	42D
F_0	*N*	5	5	7	11	6	5	10	6	8	5	23	22	20	9
	Egg	100.0	100.0	94.2	63.6	38.1	16.1	25.7	24.4	17.1	1.4	14.1	20.1	18.3	8.1
	Larvae	–	–	5.8	36.4	61.9	67.1	55.9	51.7	54.4	49.3	41.6	36.2	32.4	43.9
	Pupae	–	–	–	–	–	16.8	18.4	15.7	14.6	19.6	11.4	9.3	10.2	13.2
	Adults	–	–	–	–	–	–	–	8.2	13.9	29.7	33.0	34.4	39.1	34.8
F_1	*N*	10	5	5	5	5	5	8	7	5	5	9	13	8	12
	Egg	100.0	100.0	98.6	82.4	57.0	40.2	33.0	14.2	34.0	11.9	8.3	14.1	4.6	16.7
	Larvae	–	–	1.4	17.6	43.0	57.1	62.3	75.3	53.9	62.3	40.7	29.5	22.3	22.9
	Pupae	–	–	–	–	–	2.7	4.7	9.7	9.7	16.5	14.2	10.1	10.9	7.1
	Adults	–	–	–	–	–	–	–	0.8	2.4	9.2	36.8	46.3	62.3	53.3

Offspring on the diet surface are also included in the statistics.

numbers and peak periods for individual stages, the point when they were first observed was consistent. In the experiment, eggs, larvae, pupae, and progeny adults were first observed on 3, 9, 18, and 24 days, respectively (fig. 2). Not all females would lay eggs on the third day after introducing into the diet due to differences in growth and development among individuals. On the third day, the proportions of eggs in the gallery of F0 and F1 were 60% ($n = 5$) and 30% ($n = 10$), respectively.

Compared with the F0, the peaks of eggs and larvae for the F1 were delayed 3–6 days. In addition, both the mean number of eggs and larvae and the duration of the peak were more advantageous for the F1. The mean number of eggs for the F0 peaked at 20.8 ± 8.5 SD on the 9th day. Although the F1 peaked at 22 ± 11.3 SD eggs on the 15th day, numerous eggs were observed on 12–18 days. Larvae peaks of F0 and F1 were 15–27 days and 18–30 days, respectively. The mean number of larvae at peak for F0 and F1 were 19.5 ± 2.8 SD and 26.78 ± 4.3 SD (fig. 2, table 2).

The mean number of pupae and adult offspring in the two generations was not significantly different. Until 42 days, the number of pupae remained relatively stable, with a mean of 4.5 ± 1.3 SD and 3.1 ± 2.4 SD for F0 and F1 generations. The means of progeny adults were 8.6 ± 3.1 SD and 6.6 ± 5.1 SD for F0 and F1 generations (fig. 2, table 2).

Gallery construction

The gallery system consists of main gallery (M), branch tunnels (B), and side tunnels (S). Based on the emergence time and length of these three components, the gallery system is divided into five developmental stages (fig. 3B). The change in length of each component is shown in fig. 3A. From 3 to 42 days, the total length of the gallery system increased with the number of branches. A significant positive correlation existed between the average gallery length and the number of offspring throughout development (within 43 days) (fig. 3C). After being introduced into the tube, the female adult initially began boring the main gallery parallel to the tube wall (fig. 1D). The length of the main gallery does not show an increase from 3 to 42 days but remains relatively stable. Branch tunnels are randomly distributed on both sides of the main gallery. It began to appear from day 6 and its numbers gradually increased from 6 to 39 days (fig. 3A, table 3). During this period the surface of the diet will be covered with symbiotic fungi (fig. 1A), and the entrance to the tunnel has a large amount of cylindrical sawdusts (fig. 1B).

Within 3 days, the main gallery (M) length would gradually increase and reach 2.9 ± 0.6 SD cm (fig. 3A). Some females started oviposition on the third day. The eggs were usually detected at the end of the main gallery. Until 9 days, adult females continued to expand the main gallery to 5.6 ± 2.1 SD cm and started to excavate out the first branch tunnel (B1) in the upper middle part of the main gallery (fig. 3A, B). The first branch tunnel (B1) and second branch tunnel (B2) appeared in 12 and 18 days, respectively. Between 12–30 days, the changes in the gallery were mainly reflected in the increase of branch tunnel. The mean number of branch tunnels increased from 1.4 ± 0.5 at 12 days to 2.6 ± 0.5 at 30 days (table 3). The side tunnel was first observed on 27 days (table 3). However, not all samples showed side tunnels after 27 days, and only a tiny proportion of all dissected specimens developed side tunnels. The proportion of side branches in colonies dissected at 27, 33, 36, and 39 days were 11.8, 20, 44.4, and 20% (table 3).

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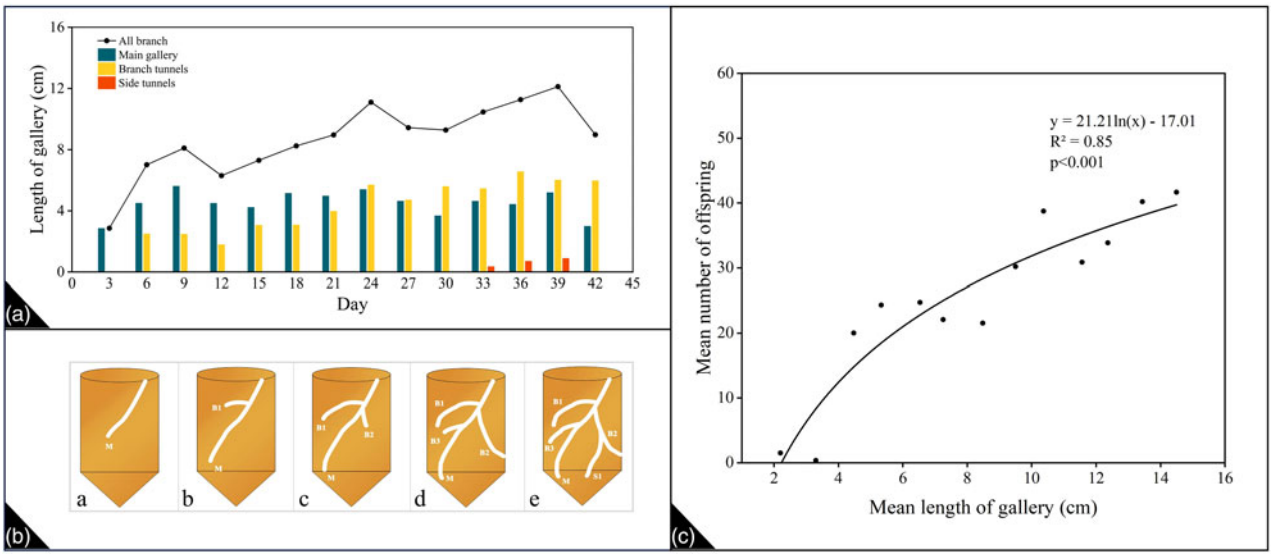

Figure 3. (a) The mean length of the main gallery, branch tunnels, and side tunnels constructed by a single foundress over time. The female beetles introduced in tubes were collected from dissecting logs. The number of colonies dissected is shown in table 3. (b) Schematic representation of five processes in the construction of *E. interjectus* gallery system. (a) Morphology of the gallery on 3rd day, only the main gallery. (b) Morphology of the gallery in 6–9 days, the first branch tunnel appears, (c) Morphology of the gallery in 12–18 days, the length of the first branch tunnel expands, and the second branch tunnel appears. (d) Morphology of the gallery on 21–30 days, three branch tunnels in colonies. (e) Morphology of the gallery on 33–42 days, side tunnel appears. (c) Mean number of adults plotted against gallery length.

Sex ratio

In the experiment of dissection, 743 beetles (adults and pupae;♀♂ = 632:111) were collected from F0 generation colonies ($n = 50$) between 4–6 weeks. The sex ratios were average 5.7:1 (♀:♂) and ranged from 0.3:1 to 19:1. Similarly, 709 beetles (adults and pupae;♀♂ = 620:89) were collected in from F1 generation colonies ($n = 54$) between 4–6 weeks. The sex ratios were average at 7:1 (♀:♂) and ranged from 2:1 to 19:1 (table 4). Female-only colonies dissected between 4–6 weeks were not used for sex ratio determination.

In the experiment of dispersal behaviour, beetles in 48 tubes of artificial diet were counted. In total, 938 beetle specimens (♀: 796; ♂: 141) were collected from tubes between 4 and 12 weeks (table 4). The offspring adult of female and male was first observed in 27 d and 25 d, respectively. According to Extreme fit, the dispersal peak of female and male adults was about 6.25 ± 0.09 SE weeks and 6.65 ± 0.30 SE weeks (fig. 4). In this study, the sex ratio of adults emerging from artificial diets was an average of 5.6:1(♀:♂) and ranged from 0.43:1 to 26:1. This value was similar to that of Xyleborini ambrosia beetle, which has an average ratio of 7.4 and 7.2% (Cooperband *et al.*, 2016). Only female offspring were found in eight tubes (17.0%) of all tubes examined.

Haplodiploid reproduction

Like other species, unfertilised females of *E. interjectus* produce unfertilised eggs that will develop into males, while fertilised females produce fertilised eggs that can develop into females. The number of offspring produced by fertilised and virgin females at 20 days was not significantly different ($P > 0.05$).

No female pupae or adults were present in the virgin female colonies before 40 days (table 5). However, of all the virgin female colonies dissected after 50 days, female pupae or adults were observed in five tubes (33%). The second generation of female pupae and adults first emerge at 53 and 57 days, respectively. This result indicates that the foundress female adult could mate with her male offspring and produce female offspring to make the family thrive.

Developmental time of each stage

Using the fungal medium (fig. 5A), we observed several changes in the development, including egg incubation, larvae moult (fig. 5B), larvae pupate (fig. 5C), and pupae eclosion. The time of discovery of eggs, larvae, and pupae was recorded as day 0 during rearing.

Table 3. Number of the branch tunnel and the side tunnel in the gallery system over time

Time (day)	3	6	9	12	15	18	21	24	27	30	33	36	39	42
N[a]	5	5	5	5	6	5	5	5	17	5	10	9	5	4
Number of branch[b]	0	1	1	1.4	1.3	1.7	2.2	2	2.1	2.6	2,5	3	3.2	2.3
Gallery with side tunnel[c]	–	–	–	–	–	–	–	–	2	–	2	4	1	–

[a]Number of galleries dissected to measure the length.
[b]Mean number of branch tunnels = total number of branch tunnels of *N*/*N*.
[c]The number of galleries with side tunnels in *N*.

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Table 4. The sex ratio measured by dispersal behaviour and dissection

	Dispersion	Dissection	
Generation	F_0	F_0	F_1
Time	4–12 weeks	4–6 weeks	4–6 weeks
Sex ratio (♀:♂)	5.6: 1 $N = 40$	5.7: 1 $N = 50$	7.0: 1 $N = 54$

The survival eggs, larvae, and pupae in the experiment were 74.19, 52.01, and 92.66%, respectively (fig. 6A). More than 25% of the larvae died in the first 6 days. About half of them were judged dead due to being undetected during observation. Between 7–13 days, the larval development process was smooth with minimal mortality. After 30 days, about 10% of the larvae showed growth retardation, and only 1–2% of them can pupate successfully.

According to observation and statistics, the development time of eggs, larvae, and pupae were 5.6 ± 0.5 SD, 17.92 ± 3.84 SD, and 5.99 ± 0.48 SD days, respectively (fig. 6B). The development time of eggs and pupae was generally concentrated in 5–7 days, and the time for larvae was focused on 13–25 days, up to 35 days. Larvae have three instars, and increasing instars are accompanied by moulting behaviour (see supplementary moult) (fig. 5D, E). Two moult peaks were found at 3.45 ± 0.64 SD and 7.92 ± 1.77 SD days (fig. 6C). The head capsule width of each instar larvae after moulting are 318.02 ± 7.38 SD, 403.01 ± 11.08 SD, and 549.54 ± 20.74 SD μm respectively (fig. 5F). 1st instar larvae are small but highly mobile and can wander the wall of Petri dishes. The development of third larvae had the most prolonged duration. The main change during this period was a rapid increase in size. A prepupal stage occurs before the larvae enter the pupal stage. The prepupae stage usually lasts 1–2 days and can be distinguished by the enlarged head, during which they typically remain stationary.

The eclosion time of male and female pupae was significantly different (fig. 6D), and the average eclosion time of male and female pupae was 5.2 ± 0.4 SD and 6.1 ± 0.4 SD days, respectively (Mann–Whitney U test: $P < 0.001$, $Z = -6.867$, $N = 22 + 94$). From the first instar larva to pupa, the fastest for males and females is 13 days, and the slowest is 22 and 35 days, respectively.

Discussion

Rearing with artificial diet

Before this study, several papers had been published on the rearing of ambrosia beetles, which describing their fecundity and other life behaviours (Weber and McPherson, 1983; Mizuno and Kajimura, 2002; Biedermann *et al.*, 2009; Maner *et al.*, 2013; Cruz *et al.*, 2018). Breeding success is a crucial indicator to judge whether laboratory breeding is successful. Using the same diet (diet-1), we found that the F1 generation was not significantly higher than that of the F0 generation in the breeding success and productivity (table 6). Perhaps it is related to their living in the same nutritional artificial diet. Identical nutritional

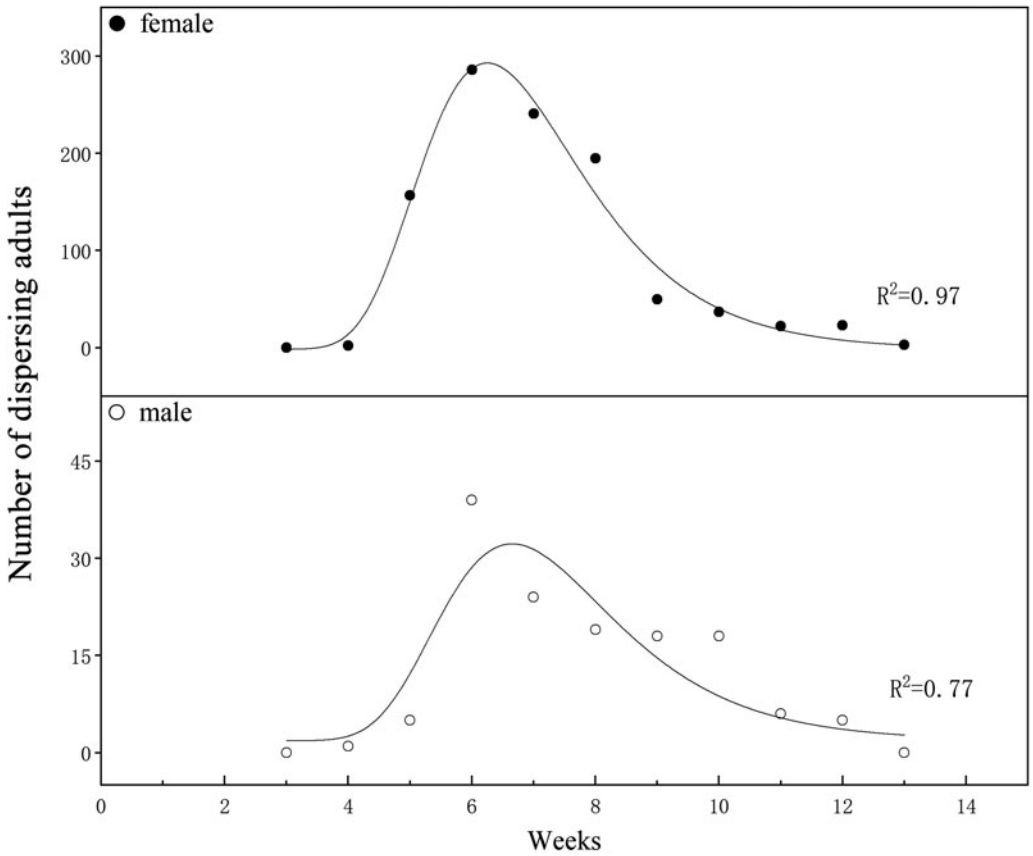

Figure 4. Dispersal peak of offspring adults. The number of dispersing beetles collected from 47 tubes of artificial diet every week. fitnlcurves (Extreme) show trends and peak periods of dispersing adults in laboratory rearing.

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Table 5. Brood productivity of fertilised and virgin females

Foundress	Period of dissection	*N*	Number of offspring per tube (mean ± SD)	Total number of pupae and new adults	
				♀	♂
Fertilised	20–30 d	12	31.3 ± 15.4[a]	54	10
Virgin	20–30 d	14	26.1 ± 19.8[a]	0	129
Virgin	30–40 d	16	26.9 ± 13.6	0	269
Virgin	50–60 d	15	21.3 ± 13.9	6 (n = 5)	234

[a]No significant difference in offspring numbers between fertilised and unfertilised females based on the *t*-test.

conditions and living environments create fungal gardens of similar abundance. For *Xyleborus glabratus*, there is no difference in the breeding success and fecundity of different origin foundress reared on the same diet (Maner *et al.*, 2013). In addition, this experiment also shows that the origin of the foundress does not seem to affect their fecundity. It may be that the nutrition of the artificial diet affects the number of offspring. Therefore, when we do not add additional nutrients to the diet (diet-2), the amount of eggs laid by the foundress is much less than that of diet-1 (table 6). Perhaps sawdust and agar initially contain trace amounts of nutrients that promote the sparse growth of symbiotic fungi. Regardless of whether living in the diet-1 or diet-2, the offspring could grow normally, develop into adults, and lay eggs again. We speculate that the foundress may be able to define nutrient abundance or scarcity and thus adjust oviposition strategies to ensure the most correct number of eggs. Furthermore, the foundress had the worst oviposition performance on a nutrient-rich but woodchip-less diet (diet-3). Perhaps females prefer to lay their eggs in wood-like structures rather than agar with added nutrients.

The peak period of F0 generation eggs and larvae is about 3 days earlier than that of F1 generation. This may be because the F0 generation lays eggs earlier than the F1 generation. Typically, foundress choose to lay eggs only after the fungal garden in the gallery has been successfully established (Peer and Taborsky, 2007). We speculate that the female adults (F0 generation) collected from the logs are in the oviposition phase and could lay eggs immediately after accessing the diet without waiting for the successful establishment of the fungal garden. By dissecting the gallery, we found that the female's job within three days was mainly to excavate the main gallery. Some females lay eggs during this process, mainly at the end of the main gallery. Although females can only lay one egg at a time, the eggs are not dispersed throughout the colony. In most cases, the eggs will be gathered into a cluster and distributed at or near the end of tunnels. We speculate that the foundress will carry the eggs and push them deeper into tunnels to ensure enough room to move. Like fertilised females, unfertilised females could excavate the gallery and lay eggs. Mizuno and Kajimura (2002) reported that fertilised females of *X. pfeili* produced more offspring than unfertilised females. In contrast, we found no significant difference in the number of offspring produced by unfertilised and fertilised

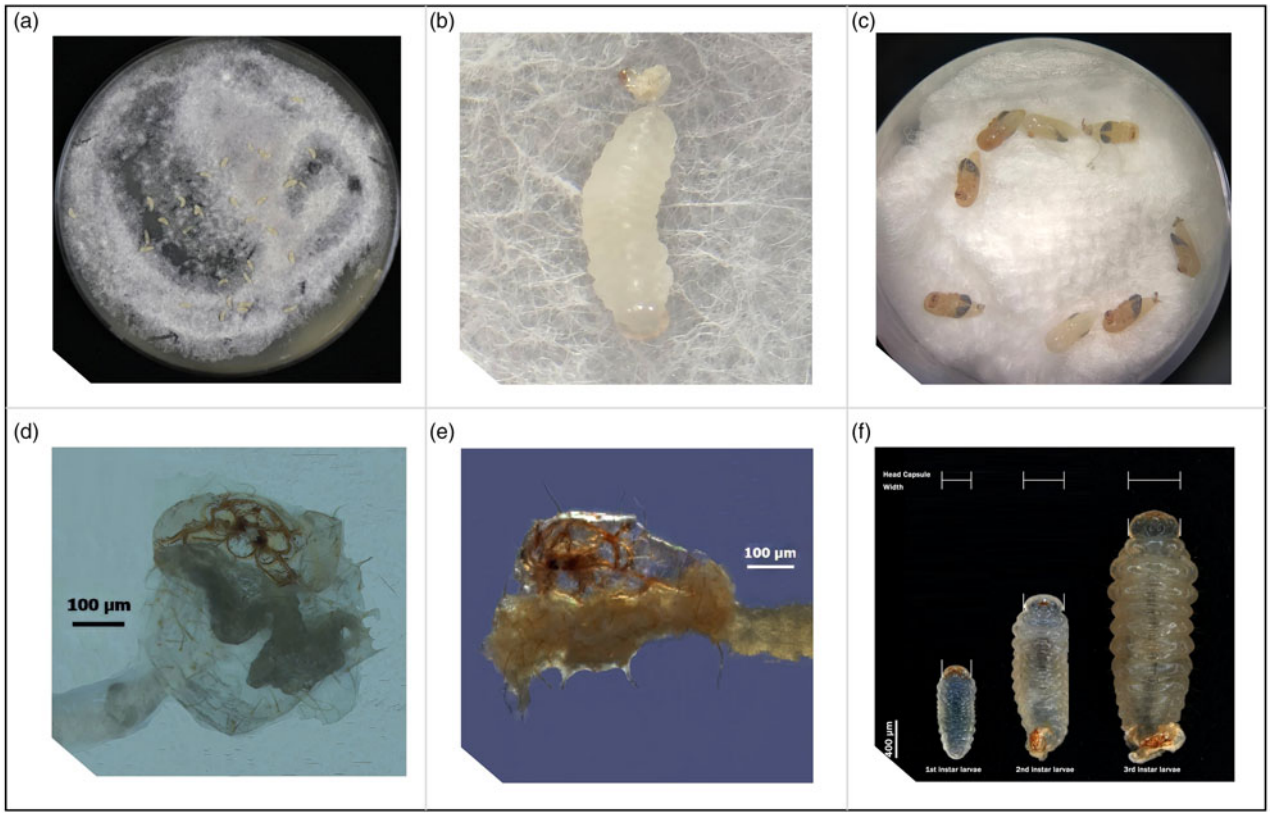

Figure 5. The record of rearing in the fungal medium. (a) Rearing larvae on day 8 with symbiotic fungi. (b) Second instar larvae that have completed moulting. Head capsule is transparent. (c) Female pupae (about to eclosion) were placed on the moist cotton cloth. (d) The moulted headshell of the first instar larvae. (e) The moulted headshell of the second instar larvae. (f) Method for measuring the head capsule of first, second and third instar larvae.

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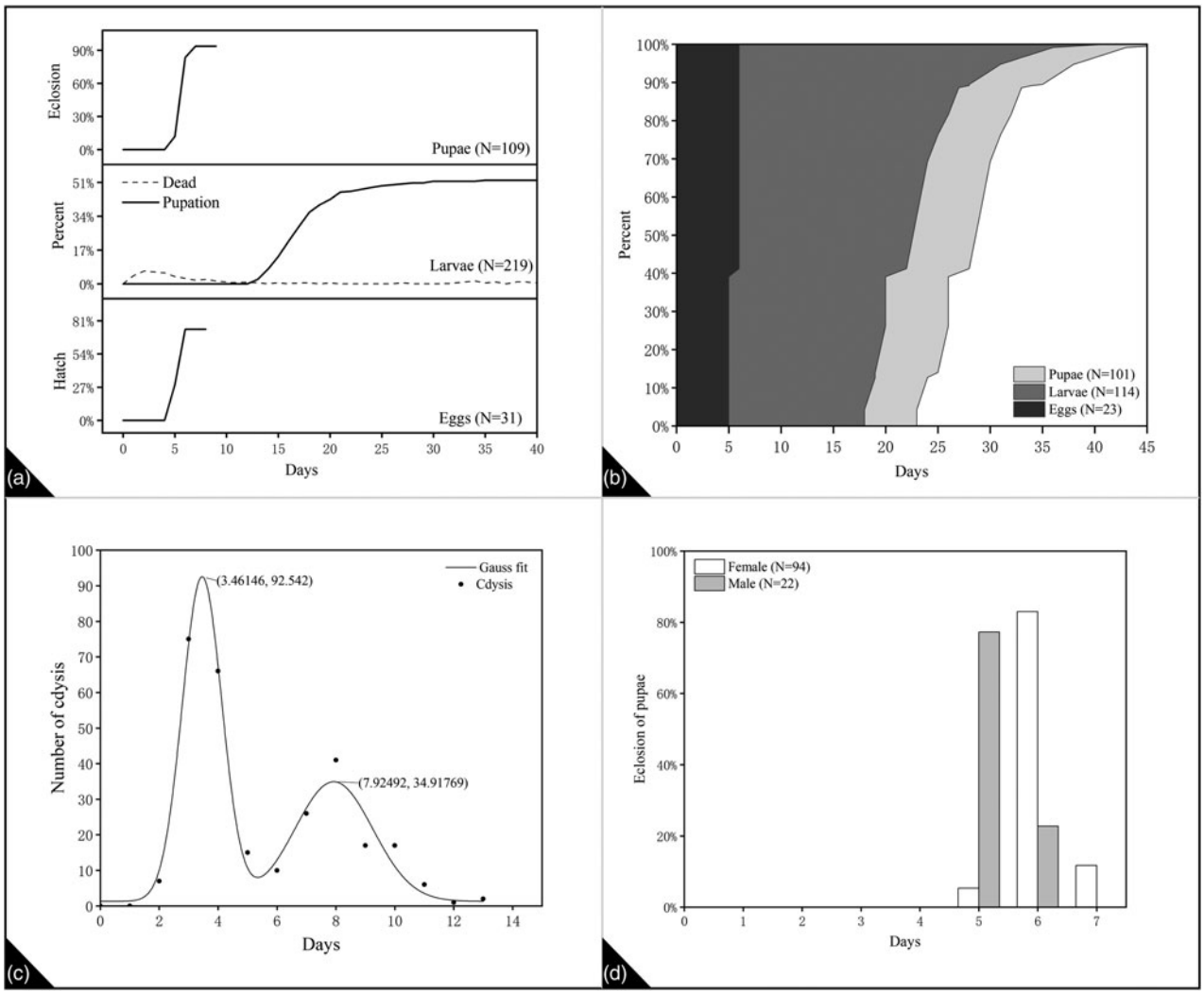

Figure 6. (a) Rearing success of eggs, larvae, and pupae in the fungal medium. (b) The life cycle of eggs, larvae, and pupae was determined by rearing in the fungal medium. (c) The peak period of moulting of first and second instar larvae (analysed by Gauss fit). (d) Eclosion peak of female and male pupae.

Table 6. Specific instructions for the three diets in the rearing process

	Diet-1		Diet-2	Diet-3
Generation	F_0	F_1	F_1	F_1
Dissection	9–43 days	33–41 days	20th, 78th day	20th day
No. of tubes	225	61	25	20
No. of offspring	33.3%	21.3%	68%	95%
Offspring dead	10.7%	11.5%	–	–
Successful	56%	67.2%[a]	32%	5%[a]
Productivity* per successful tube mean ± SD	25.7 ± 18.7 (n = 61)	21.0 ± 13.8[b] (n = 41)	4.9 ± 2.8[b]	–

[a]Significantly different between Diet-2 and Diet-3 based on the χ^2 test ($P < 0.05$).
[b]Significantly different in productivity between Diet-1 and Diet-2 based on the Mann–Whitney U test ($P < 0.05$).
*To facilitate comparison of the fecundity of F0 and F1 generations in diet-1, only data from 33–41 day dissections were used for statistics.

females of *E. interjectus*. The genes of xyleborine species may determine this difference.

For *E. interjectus*, the productivity of the foundress does not appear to be affected by the origin and fertilisation. During the rearing process, the significant difference in the fecundity of foundress is caused by the different substrate of the diet (diet-1 and diet-2). Nuotclà *et al.* (2021) reported differences of *Xyleborinus saxesenii* in the fecundity and the fungus garden composition in dry and humid substrate. Perhaps for *E. interjectus*, the fecundity of the foundress also changes with the fungus garden composition. But if the fungi all enrich nutrients from the same substrate (diet-1), the fecundity of the foundress will not differ significantly even if the origin of the foundress is different.

The fluctuating trend in the length of the gallery system serves as the basis for determining the potential involvement of adult progeny in its construction. Like the gallery without progeny adults (12–24 days), the length of the gallery system with progeny adults (27–39 days) exhibits a consistent and similar upward trend (fig. 3A). Perhaps the offspring adults will not participate in the construction of the gallery system, and they will take

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more responsibility for tending and maintenance. In addition to gallery length, the number of eggs does not increase significantly after the emergence of adult offspring (fig. 2). Perhaps in the colonies, only one female (foundress) in the gallery is responsible for excavating the tunnels and oviposition. The offspring adults do not seem to lay eggs in the original gallery but instead disperse to find hosts to excavate new gallery and oviposit. As Peer and Taborsky (2007) mentioned, the ambrosia beetle exhibit high sociality, and the offspring adults will delay dispersal and cooperate to take care of the fungus gardens and brood. We found that the dispersal peak of offspring males was later than that of females in laboratory rearing (fig. 4). This result is coordinated with the hypothesis of Biedermann. Males may not participate in social activities, and they inseminate an entire brood of sisters through high sexual activity (Biedermann, 2010).

Rearing with fungal medium

Fungal medium rearing changes the natural living environment of the ambrosia beetle, making it possible to observe instars and social behaviour. In previous studies, observation experiments on glass tubes were extensively performed to determine developmental timing and social behaviour (Biedermann, 2010; Biedermann and Taborsky, 2011). Perhaps, the method of fungal medium rearing in this experiment can provide novel inspiration and ideas for the behavioural observation of the ambrosia beetle.

From the survival perspective, rearing from first-instar larvae to pupae had a poor performance of 52.01%. In our opinion, there are three main reasons why larvae are difficult to rear (1) they need the right environment, but also need to meet their nutritional requirements; (2) first instar larvae are small and active, they like to escape and can easily be overlooked during the counting process; (3) Although the maximum developmental time of larvae is 35 days, many larvae (20%) are developmentally arrested after 30 days and do not pupate successfully. This phenomenon has not been reported in observations of ambrosia beetle species. It is unclear whether a nutritional or genetic cause is responsible for developmental arrest.

It is impossible to observe larval moulting behaviour in artificial diet and accurately sample different instars larvae after moulting. The rearing of fungal medium may help determine the larval instars of different ambrosia beetles. However, in the process of rearing larvae using fungal medium, how to avoid larval escape behaviour may be a question worth thinking about.

Conclusion

This study investigates the developmental duration and biological behaviour of *E. interjectus* under laboratory rearing conditions using artificial diet and fungal medium. The development time of each stage determined by fungal medium rearing is consistent with the time observed by dissecting the diet. During development, females will expand their branches before the peak of each developmental stage to ensure an adequate supply of food resources. However, the offspring adults do not participate in the construction of the gallery system after they emerge, and they disperse from the natal gallery after fertilisation. Haplodiploid reproduction does not affect the number of offspring they produce. Food resources (fungi) and space (gallery length) could determine offspring population quantity. The composition and abundance of fungal gardens may better explain the fecundity of foundress and the development of offspring, which is also one of our subsequent research directions. We will further explore the impact of fungal communities under different nutrients on the growth and development of offspring, especially the impact of fungal gardens on larval growth and development. Research on fungal gardens can explain the biology of *E. interjectus* from diverse perspective. Changing the fungus garden composition to reduce food resources may be an effective way to control the ambrosia beetle.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485324000233

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Competing interests. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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