

# Fungus Cultivation by Ambrosia Beetles: Behavior and Laboratory Breeding Success in Three Xyleborine Species

PETER H. W. BIEDERMANN,<sup>1,2,3</sup> KIER D. KLEPZIG,<sup>3</sup> AND MICHAEL TABORSKY<sup>1</sup>

Environ. Entomol. 38(4): 1096–1105 (2009)

**ABSTRACT** Fungus cultivation by ambrosia beetles is one of the four independently evolved cases of agriculture known in animals. Such cultivation is most advanced in the highly social subtribe Xyleborina (Scolytinae), which is characterized by haplodiploidy and extreme levels of inbreeding. Despite their ubiquity in forests worldwide, the behavior of these beetles remains poorly understood. This may be in part because of their cryptic life habits within the wood of trees. Here we present data obtained by varying a laboratory breeding technique based on artificial medium inside glass tubes, which enables behavioral observations. We studied species of the three most widespread genera of Xyleborina in the temperate zone: *Xyleborus*, *Xyleborinus*, and *Xylosandrus*. We raised several generations of each species with good breeding success in two types of media. The proportion of females of *Xyleborinus saxesenii* Ratzeburg producing offspring within 40 d depended significantly on founder female origin, which shows a transgenerational effect. Labor-intensive microbial sterilization techniques did not increase females' breeding success relative to a group of females shortly treated with ethanol. Gallery productivity measured as the mean number of mature offspring produced after 40 d varied between species and was weakly affected by the type of medium used and foundress origin (field or laboratory) in *X. saxesenii*, whereas different preparation and sterilization techniques of the beetles had no effect. Behavioral observations showed the time course of different reproductive stages and enabled to obtain detailed behavioral information in all species studied. We propose that the laboratory techniques we describe here are suited for extensive studies of sociality and modes of agriculture in the xyleborine ambrosia beetles, which may yield important insights into the evolution of fungal agriculture and advanced social organization.

Die Pilzzucht der Ambrosiakäfer stellt eine von vier unabhängigen Entwicklungen von Ackerbau im Tierreich dar. Im hochsozialen Untertribus Xyleborina (Scolytinae), für den Haplodiploidität und extreme Inzucht charakteristisch sind, ist die Pilzzucht am stärksten ausgeprägt. Die in Tunnelsystemen im Kernholz von Bäumen lebenden Ambrosiakäfer sind trotz ihrer weltweiten Verbreitung und Häufigkeit noch kaum untersucht und ihr Verhalten ist weitgehend unbeschrieben. In diesem Artikel präsentieren wir eine Labor-Zuchttechnik in künstlichem Nährmedium in Glasröhrchen, die es erlaubt, das Verhalten der Käfer in ihren Galerien zu beobachten. Wir studierten drei Arten der am weitesten verbreiteten Gattungen *Xyleborus*, *Xyleborinus* und *Xylosandrus*. Unsere Bruttechnik ermöglichte die Zucht mehrerer Generationen aller drei Arten mit gutem Bruterfolg, wobei die Wahrscheinlichkeit einer erfolgreichen Brut von *Xyleborinus saxesenii* Ratzeburg von der Vorgeschichte des Gründerweibchens, nicht jedoch von der Sterilizationstechnik vor dem Einsetzen der Käfer in das Nährmedium bestimmt wurde. Die Galerieproduktivität unterschied sich zwischenartlich, und hing bei *X. saxesenii* in geringem Mass vom Brutmedium und der Herkunft (Feld oder Labor) der Käfer ab, wohingegen die Sterilizationstechnik und die Vorgeschichte des Gründerweibchens auf diesen Parameter keinen Einfluss hatte. Die hier vorgestellten Verfahren ermöglichen detaillierte Untersuchungen am Sozialverhalten und an den Ackerbaumethoden von xyleborinen Ambrosiakäfern, was wesentlich zum Verständnis evolutiver Mechanismen von Pilzzucht und höherer Sozialität im Tierreich beitragen kann.

**KEY WORDS** artificial medium, symbiosis, insect agriculture, cooperative breeding, biological invasions

Ambrosia beetles in the subfamilies Scolytinae and Platypodinae (Curculionidae: Coleoptera) exhibit di-

verse life histories and are highly useful as model systems for evolutionary studies of sociality, behavior, and interspecific relations. Ambrosia beetles are a polyphyletic group defined by living in nutritional symbiosis with ambrosia fungi, which are often species of the order Ophiostomatales (Ascomycota). Embedded in a microbial complex of other fungal associates,

<sup>1</sup> Institute of Ecology and Evolution, Division Behavioral Ecology, University of Berne, Baltzerstrasse 6, 3012 Bern, Switzerland.

<sup>2</sup> Corresponding author, e-mail: peterbiederm@students.unibe.ch.

<sup>3</sup> U.S. Forest Service, Southern Research Station, 200 WT Weaver Blvd., Asheville, NC 28804.

yeasts, and bacteria (Haanstad and Norris 1985), these fungi are cultivated in tunnels excavated by the beetles in the xylem (sapwood and/or heartwood) of dying or recently dead trees (Beaver 1989, Kirkendall et al. 1997). Nutritional symbioses with fungi have evolved multiple times in arthropods (Batra 1979), and there are at least seven origins of this habit in the Scolytinae (Farrell et al. 2001, Sequeira and Farrell 2001). However, fungus tending and gallery maintenance behaviors, i.e., active fungiculture (Batra and Michie 1963, Kirkendall et al. 1997, Biedermann 2007), are only exhibited by ambrosia beetles. Although virtually unstudied, ambrosia beetles are now regarded as one of the four agricultural groups in the animal kingdom (along with humans, macrotermite termites, and attine ants; Farrell et al. 2001, Mueller et al. 2005). In the other three groups, agriculture is associated with some form of sociality, which is assumed to apply to ambrosia beetles as well (Kent and Simpson 1992, Kirkendall et al. 1997, Mueller et al. 2005, Peer and Taborsky 2007).

From an evolutionary point of view, the Xyleborina comprise an especially interesting group to study among the ambrosia beetles. This subtribe is predisposed to advanced forms of sociality and fungiculture as a result of haplodiploidy and obligate inbreeding (Peer and Taborsky 2007). Female offspring mate with their brothers (sex ratio around 1:20 for M:F) in the natal gallery (Peer and Taborsky 2004, 2005). Therefore, females are similarly related to their sisters, own offspring, and all other offspring produced by colony members, which increases the potential to gain indirect fitness benefits by cooperative brood care and fungiculture. Indeed, mature females delay dispersal from their natal nest depending on offspring numbers (*X. saxesenii*; Peer and Taborsky 2007). The resulting overlap between generations has been regarded as a precondition for cooperative interactions and a first step toward the evolution of higher sociality (Gadagkar 1990, Queller 1994) and probably also fungiculture (Mueller et al. 2005). Despite the general interest in evolutionary transitions toward eusociality, hitherto Xyleborina have hardly been studied: developmental periods are largely unknown, overlapping generations have not been observed in the laboratory, and experimental studies of effects of colony size and composition are yet lacking. This lack of information is presumably mainly caused by the difficulties of observations and laboratory rearing.

The aforementioned obligate association with nutritional ambrosia fungi complicates laboratory rearing, because culture conditions must suit both beetles and fungi (Batra 1985). The beetle–fungus association in Xyleborina is maintained by females that transfer spores of species specific ambrosia fungi and potentially other associated microorganisms (the microbial complex) either in the gut or in selective spore carrying organs (mycetangia or mycangia; Francke-Grosman 1956, 1963, 1975; Batra 1963) from their natal nest to their newly founded galleries (vertical transmission). Once within the galleries, the females do not lay eggs before feeding on ambrosia fungi, the

sole food source for the remainder of the beetles' life cycle (French and Roeper 1975, Kingsolver and Norris 1977b). A successful initiation of the fungus garden is crucial for breeding success. Nevertheless, fungiculture fails at fairly high rates. In *X. saxesenii* Ratzeburg, for example, only 20% of founded fungus gardens are successful (Fischer 1954, Biedermann 2007, Peer and Taborsky 2007). The most obvious reasons for failure are (1) unsuitable substrate and conditions for fungus growth (e.g., type of wood, humidity, level of fermentation), (2) the presence of antagonistic organisms (e.g., other fungi, bacteria, mites), and (3) loss of ambrosia fungi during dispersal. To address these problems in the laboratory, various media (Norris and Baker 1969, Roeper et al. 1980a, Mizuno and Kajimura 2002), sterilization techniques (Peer and Taborsky 2004, 2005) including "fungitoxic meridic medium" (Norris and Chu 1985), and various insect collection methods have been tested (Francke-Grosman 1963, Batra 1985). However, none of the rearing techniques mentioned above were developed for behavioral studies, nor have any of them been used for more than one ambrosia beetle species. As a result, there is little data on how nutritional medium and beetle treatment affects breeding success (for exceptions, see French and Roeper 1972, Mizuno and Kajimura 2002).

In this study we sought to develop and test rearing techniques that allow behavioral observations and manipulations of individual beetle numbers. In addition, we wished to be able to alter fungus species composition to allow comparative and experimental studies on the evolution of social behavior and fungiculture in ambrosia beetles. Our model system is the scolytine subtribe Xyleborina (including *Xyleborus*, *Xyleborinus*, and *Xylosandrus*), which is predisposed for group living and social evolution by environmental factors (e.g., difficulties in host finding and successful establishment of fungus gardens) and genetical conditions (haplodiploidy, inbreeding). Because of interspecific variation in these predisposing factors, Xyleborina are a very promising group to find gradual evolution toward higher sociality and fungiculture. Therefore, we aimed to test the general suitability of a newly adapted standard technique by rearing one representative species per genus (*X. affinis* Eichhoff, *X. saxesenii* Ratzeburg, and *X. germanus* Blandford) and comparing their breeding success (i.e., the proportion of females producing offspring within 40 d) and productivity (i.e., the mean number of mature offspring produced after 40 d). Among those three, *X. saxesenii* is regarded to exhibit the highest sociality. Mature daughters delay their dispersal from the natal gallery in the field (Peer and Taborsky 2007), and adults, offspring, and fungus gardens are all found in close contact in one common brood chamber (Biedermann 2007). However, this species is the least amenable to laboratory rearing. We therefore tried to increase breeding success by optimizing the medium and testing effects of beetles' origin and different beetle sterilization techniques before rearing. These results may also help to gain insights into potential transgenerational effects (e.g., of group size) and into details of the beetle–ambrosia

Table 1. Origin of study species and their treatment before and during breeding

|                                  | <i>Xyleborus affinis</i>                  | <i>Xylosandrus germanus</i>             | <i>Xyleborinus saxesenii</i>  |
|----------------------------------|---|---|---|
| Place of collection              | Pineville/USA, 41 m asl 31°20', 92°24'    | Bern/CH, 560 m asl 46°95', 7°31'        | Bern/CH, 560 m asl 46°95', 7°31'  |
| Mode of collection               | Ethanol baited traps/laboratory galleries | Ethanol-baited traps/dissection of logs | Ethanol-baited traps/dissection of logs/laboratory galleries  |
| Treatments before breeding       | Ethanol-sterilization                     | Ethanol-sterilization                   | Ethanol sterilization and refrigeration/several days in fungitoxic medium/sterilization after Francke-Grosman |
| Medium                           | Standard (SM)                             | Standard (SM)                           | Standard (SM)/modified (MM)   |
| Number of laboratory generations | Multiple                                  | One                                     | Multiple  |

CH-Switzerland; asl-above sea level.

fungus relationship. The large common brood chambers, if built next to the tube glass, may also allow for collecting the first data on *X. saxesenii*'s developmental period and overlapping generations in the laboratory.

### Materials and Methods

**Study Species.** The subtribe Xyleborina (Scolytinae, Curculionidae) comprises ~1,400 mostly tropical species belonging to 26 genera (Hulcr et al. 2007). Among them *Xyleborus*, *Xylosandrus*, and *Xyleborinus* are the only genera that are also ubiquitous in the temperate zones around the world. The most widespread species in the subtribe are *X. affinis* Eichhoff, *X. germanus* Blandford, and *X. saxesenii* Ratzeburg. *X. affinis* is native to the American tropics and subtropics, whereas *X. germanus* and *X. saxesenii* are native to temperate Eurasia. Because all three are perfectly adapted to inbreeding (Peer and Taborsky 2005), they are successful invasive species and frequently found as exotics (Jordal et al. 2001), causing varying degrees of economic damage (Rabaglia et al. 2006, Fraedrich et al. 2008). Tree damage is often caused by beetle vectored microorganisms ("the microbial complex"; Haanstad and Norris 1985). The actual ambrosia fungi, however, are usually nonpathogenic to plants (Beaver 1989). The mutualistic fungi of our study species are *Cephalosporium pallidum* Verrall for *X. affinis* (Verrall 1943), *Ambrosiella hartigii* Batra for *X. germanus* (Weber and McPherson 1984), and *A. sulfurea* Batra for *X. saxesenii* (Batra 1966, Francke-Grosman 1975). Adults of all Xyleborina are exclusively mycetophagous, whereas the larvae of most species of *Xylosandrus* and *Xyleborinus* are xylomycetophagous. This means that, with the exception of larvae of the latter two genera, which frequently ingest fungus-infested wood (Biedermann 2007), all other Xyleborina larvae and adults seem to feed solely on fungal tissues. This difference is responsible for the characteristic brood chambers made by larvae that are only found in the tunnel systems of *Xylosandrus* and *Xyleborinus* (Roper 1995, Biedermann 2007). All three species colonize a wide variety of dying or recently dead deciduous tree species (Wood 1982, Pfeiffer 1995).

**Preparation of Artificial Media.** Using an aseptic technique (for all the steps listed below), we filled sterile glass tubes (18 by 150-mm culture tubes; Bellco

Glass, Vineland/NJ) with two types of artificial media. The standard medium (SM) consisted of 0.35 g streptomycin, 1 g Wesson's salt mixture, 5 g yeast, 5 g casein, 5 g starch, 10 g sucrose, 20 g agar, 75 g beech or oak tree sawdust, 2.5 ml wheat germ oil, and 5 ml 95% ethanol (Peer and Taborsky 2004, 2005; modified from Norris and Chu 1985). The modified medium (MM) reported here (we tried out several modifications of the standard medium and found by trial and error the "modified medium" reported here to render the highest success) contained the same components as the SM, but the following in different concentrations: Wesson's salt mixture 1.25 g, casein 10 g, agar 30 g, beech tree sawdust 200 g, and sucrose 5 g. Additionally, the modified medium contained 2.5 ml peanut oil. Both media were prepared by first mixing all dry ingredients and adding 500 (SM) or 580 ml (MM) of deionized water. The SM mixture was autoclaved for 20 min at 124°C, and the hot medium was poured into sterile tubes and covered immediately with plastic caps (Bellco Glass kap-uts, Vineland/NJ). This amount of medium was enough to fill ~40 (SM) or 80 (MM) tubes (18 by 150 mm). The MM mixture was poured directly into the test tubes and autoclaved within the tubes at the same time and temperature conditions. After the medium cooled down, we scratched its surface with a sterilized spatula to facilitate gallery initiation by founder females. We closed the tubes with sterile plastic caps and left them to dry for 4–5 d.

**Beetle Collection and Standard Rearing Conditions.** Females were collected by catching dispersing females in two different ways. We either caught dispersing females with ethanol (95%)-baited live traps or by dissecting logs with active galleries (Table 1). Galleries were found by inspecting logs from weakened or freshly dead host trees exhibiting small bore holes (diameter ~1 mm) exuding fine boring dust or compact cylinders of frass (Batra 1985).

*Xyleborus affinis* females were collected from oak logs in Pineville, LA, from July to September 2007. Females of *X. germanus* and *X. saxesenii* were collected from beech logs and trapped near Bern, Switzerland, during summer 2005 (Table 1). Additionally, we initiated new breeding units using females from our laboratory cultures and reared them for several successive generations (except *X. germanus*). After collection in the field, the females were brought di-

rectly to the laboratory for treatment on a sterile bench (some exceptions for *X. saxesenii*, see below). There, females were quickly surface sterilized with 70% ethanol and deionized water and placed individually into the glass tubes. This surface sterilization likely reduced the load of phoretically (and unintentionally) vectored microorganisms (e.g., molds). These "contaminants" would likely establish more easily in our homogenic artificial media than under natural conditions (where beetles self surface-sterilize through boring bark rich in fungitoxins and other antibiotics substances; Berryman 1989). Tubes were subsequently capped and stored horizontally in darkness (wrapped in paper, but exposing the entrance to light) under room temperature ( $\approx 25^{\circ}\text{C}$ ,  $\approx 50\%$  humidity).

**Treatments of *X. saxesenii* to Increase Breeding Success.** The success of our standard breeding method for *X. saxesenii* females was very low (see Results section). We therefore tried to optimize the culture medium and pretreatment of females before they were introduced into the breeding chambers (see Table 1). (1) One group of females ( $N = 56$ , from 10 galleries) was stored in the refrigerator for 2–7 d ( $\approx 5^{\circ}\text{C}$ ; also convenient for practical reasons if it has no negative influence on breeding success). (2) A group of females ( $N = 24$ , from six galleries) was allowed to bore within fungitoxic medium for 2–4 d. This medium was similar to SM, but with the addition of 1 ml of sorbic acid (60%) before autoclaving. During the first days of boring through this medium, beetles lose all fungi attached to their body surface. If they are allowed to bore for longer, they also lose their internal fungal mutualists (Norris and Chu 1985). (3) The third group ( $N = 29$ , from seven galleries) was treated with Francke-Grosmann's surface sterilization method (Francke-Grosmann 1956, Batra 1985). These beetles were kept on sterile moist filter paper in a petri dish for 12 h, before they were transferred to sterile dry filter paper in a second petri dish and kept for another 12 h. This procedure was repeated three times; it does not affect internal mutualistic fungi (Francke-Grosmann 1956, 1963, 1975).

**Measurements and Observations.** Breeding success and gallery productivity was compared between species, collection methods, medium types, and treatments before breeding. Additionally, in *X. saxesenii*, we checked for potential effects of foundress origin (i.e., the productivity of the foundress' natal gallery) on breeding success and gallery productivity. Breeding success was defined as the percentage of females producing offspring within 40 d among all females introduced experimentally to tubes. Gallery productivity was determined as the mean number of mature offspring produced after 40 d (only successful galleries were included).

In *X. saxesenii*, we also determined the duration of different life stages within the galleries by daily counting the number of individuals in different life stages: first larval instars, second/third larval instars, pupae, teneral females (brown; not fully sclerotized), mature females (black; fully sclerotized), and males. Males are

morphologically distinct from females; they are smaller and never fully sclerotize (for details, see Fischer 1954). The duration of these life stages was determined in all well-observable galleries ( $N = 5\text{--}12$  galleries, depending on life stage). Finally, we made observations of *X. saxesenii* behavior to determine the applicability of our technique for behavioral studies in Xyleborina and to document brood and fungus initialization by the foundress.

**Statistics.** Data were not normally distributed, so we used nonparametric statistics. Variations in breeding success and gallery productivity were tested by Kruskal-Wallis ANOVAs for multiple samples and Mann-Whitney *U* tests for pairs of two samples. When we bred females from the same natal galleries under two different treatments, we used the Wilcoxon matched-pairs signed-ranks test. We used Spearman rank correlation analyses to test for relationships between the breeding success of foundresses and the productivity of their natal galleries. Analyses were performed with SPSS (Release 14.0; SPSS, Chicago, IL).

## Results

**Breeding Success of the Study Species in Standard Medium.** Breeding success differed between species [Kruskal-Wallis analysis of variance (ANOVA):  $\chi^2(2) = 23.345$ ,  $P < 0.001$ ,  $N = 49$ ] but was independent of collection mode in *X. saxesenii* Ratzeburg [Kruskal-Wallis ANOVA:  $\chi^2(2) = 2.981$ ,  $P = 0.225$ ,  $N = 34$ ; Table 2]. The same may have been true for *X. affinis* Eichhoff, although the sample size was too small for a statistical test in this species. In a post hoc comparison, we found that, for all collection modes combined, the breeding success of *X. saxesenii* in SM was significantly lower than that of *X. affinis* (Mann-Whitney *U* test:  $Z = -5.065$ ,  $P < 0.001$ ,  $N$  [galleries of *X. saxesenii* versus *X. affinis*] = 34 + 8) but not different from *X. germanus* Blandford (Mann-Whitney *U* test:  $Z = -0.789$ ,  $P = 0.575$ ,  $N$  [*X. saxesenii* versus *X. germanus*] = 34 + 7). The low breeding success of *X. saxesenii* compared with *X. affinis* was also reflected in a lower gallery productivity of males (Mann-Whitney *U* test:  $Z = -2.299$ ,  $P = 0.022$ ,  $N$  [*X. saxesenii* versus *X. affinis*] = 7 + 94) and females ( $Z = -3$ ,  $P = 0.003$ ,  $N = 7 + 94$ ). We did not measure the productivity of *X. germanus* galleries.

**Breeding Success of *X. saxesenii* in Modified Media and Under Various Treatments.** There was a trend for a higher breeding success of *X. saxesenii* reared in MM than in SM (Table 2). This was true both for foundresses from the same natal gallery (Wilcoxon test:  $Z = -1.82$ ,  $P = 0.069$ ,  $N = 9$ ) and independent of origin (Mann-Whitney *U* test:  $Z = -1.935$ ,  $P = 0.051$ ,  $N = 34 + 60$ ). Breeding success in MM tended to be higher in foundresses from laboratory galleries than in those from field galleries (Mann-Whitney *U* test:  $Z = -1.939$ ,  $P = 0.053$ ,  $N = 21 + 39$ ). None of the other treatments (refrigeration, meridic medium, FG sterilization) had any significant effect on the breeding success in either medium. However, because of apparent loss of mutualistic fungi, some females could not establish fungal

**Table 2.** Breeding success and gallery productivity in three species of *Xyleborina* in standard and modified medium dependent on the mode of collection of foundresses

|                      | <i>Xyleborus affinis</i>             | <i>Xylosandrus germanus</i>          | <i>Xyleborinus saxesenii</i>         |   |
|----------------------|--------------------------------------|--------------------------------------|--------------------------------------|---|
| Medium               | Standard                             | Standard                             | Standard                             | Modified                                |
| Breeding success     |                                      |                                      |                                      |   |
| Dissection of logs   | 90.6% ( $N_1 = 32/1$ )               | Not tested                           | 4.1% ( $N_1 = 73/16$ )               | 11.5% ( $N_1 = 61/21$ )                 |
| Field trapping       | Not tested                           | 28.6% ( $N_1 = 7/7$ )                | 0% ( $N_1 = 14/6$ )                  | Not tested                              |
| Laboratory gallery   | 85.7% ( $N_1 = 77/7$ )               | Not tested                           | 7.2% ( $N_1 = 55/12$ )               | 23.9% ( $N_1 = 347/39$ )                |
| Total                | 87.2% <sup>b</sup> ( $N_1 = 109/8$ ) | 28.6% <sup>a,b</sup> ( $N_1 = 7/7$ ) | 4.4% <sup>a</sup> ( $N_1 = 142/34$ ) | 22.1% <sup>a,b</sup> ( $N_1 = 408/60$ ) |
| Gallery productivity | 19.4 ♀ (0–86) <sup>c</sup>           | Not tested                           | 7 ♀ (0–23) <sup>d</sup>              | 12 ♀ (0–87) <sup>d</sup>                |
|                      | 0.96 ♂ (0–6) <sup>e</sup>            |                                      | 0.29 ♂ (0–1) <sup>f</sup>            | 1.66 ♂ (0–51) <sup>e,f,g</sup>          |
|                      | $N_2 = 95$                           |                                      | $N_2 = 7$                            | $N_2 = 89$                              |

Breeding success, as the percentage (%) of individuals that successfully produced a brood after 40 d. Gallery productivity, as the average number of female and male adult offspring (min – max) produced in all successful galleries after 40 d. Statistical analyses were made on the gallery level.

<sup>a,b</sup> Different letters mark significant differences in breeding success. Mann-Whitney  $U$  tests,  $P < 0.05$ .

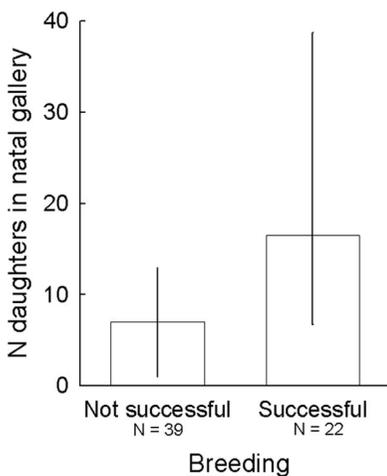
<sup>c,d,e,f</sup> Different letters mark significant differences in gallery productivity; Mann-Whitney  $U$  tests,  $P < 0.05$ .

<sup>g</sup> There were three galleries with only male offspring.

$N_1$ , individuals tested/no. of galleries or traps from which individuals originated;  $N_2$ , Number of successfully founded galleries tested, independent of collection mode.

gardens and hence did not produce broods after the meridic medium treatment. Gallery productivity of males (Mann-Whitney  $U$  test:  $Z = -1.594$ ,  $P = 0.111$ ,  $N = 7 + 89$ ) and females ( $Z = -0.698$ ,  $P = 0.485$ ,  $N = 7 + 89$ ) did not differ significantly between MM and SM.

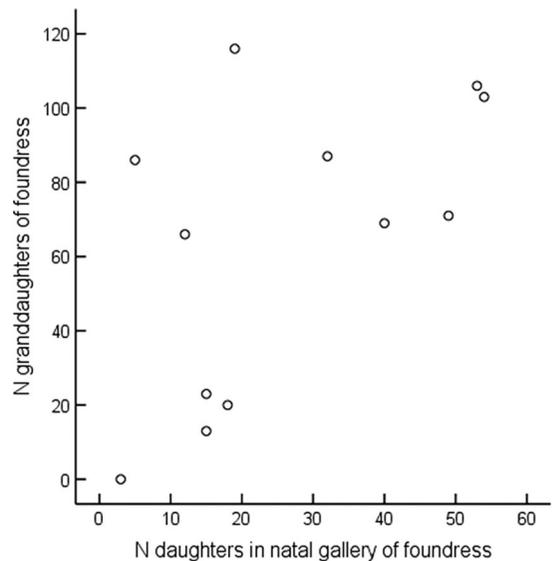
**Effects of the Foundress' Natal Gallery Conditions on Future Fitness in *X. saxesenii*.** Female breeding success was positively correlated with the number of sisters that had been produced in their natal gallery (=natal gallery productivity; Spearman ranks test:  $r_s = 0.452$ ,  $P < 0.001$ ,  $N = 61$ ). Natal gallery productivity was significantly higher for successful than for unsuccessful foundresses (Mann-Whitney  $U$  test:  $Z = -3.955$ ,  $P < 0.001$ ,  $N = 39 + 22$ ; Fig. 1). In contrast, foundress gallery productivity was not related to natal gallery productivity (Spearman ranks test:  $r_s = 0.128$ ,



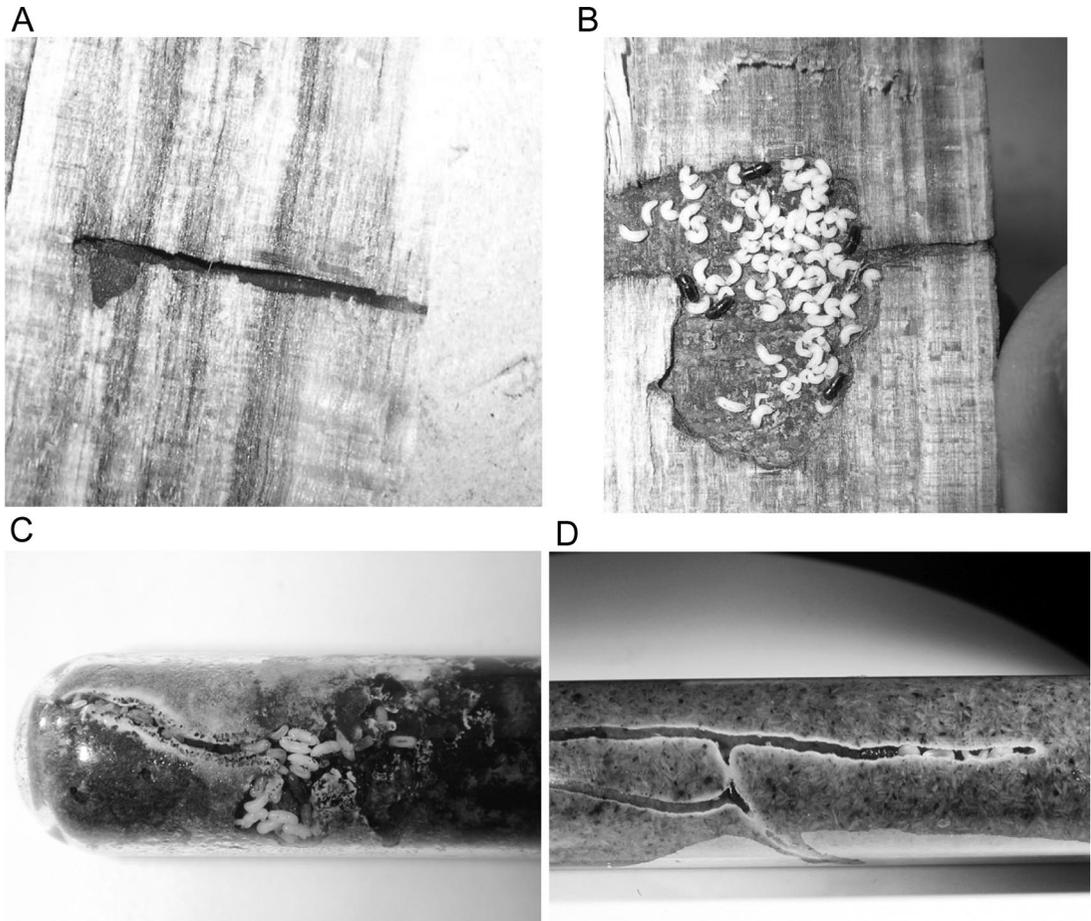
**Fig. 1.** Relationship between the success of *X. saxesenii* foundresses and the number of daughters in their natal gallery. Successful foundresses originated from larger galleries ( $P < 0.001$ ;  $N = 39 + 22$ ; Mann-Whitney  $U$  test), irrespective of origin (field or laboratory) and brood medium (SM and MM). Medians and quartiles are shown.

$P > 0.05$ ,  $N = 40$ ). The number of granddaughters of a foundress (a direct measure of fitness, combining breeding success and daughters' gallery productivity) was positively correlated with her natal gallery productivity (Spearman ranks test:  $r_s = 0.637$ ,  $P = 0.026$ ,  $N = 12$ ; Fig. 2).

**Behavioral Observations, Gallery Phenology, and Developmental Period of *X. saxesenii*.** All three species constructed large portions of their galleries (brood chambers and tunnels) alongside the wall of the glass tubes. This greatly facilitated behavioral observations of all life stages. After females were introduced onto the surface of the medium, they started to bore a tunnel perpendicular to the surface. At a depth



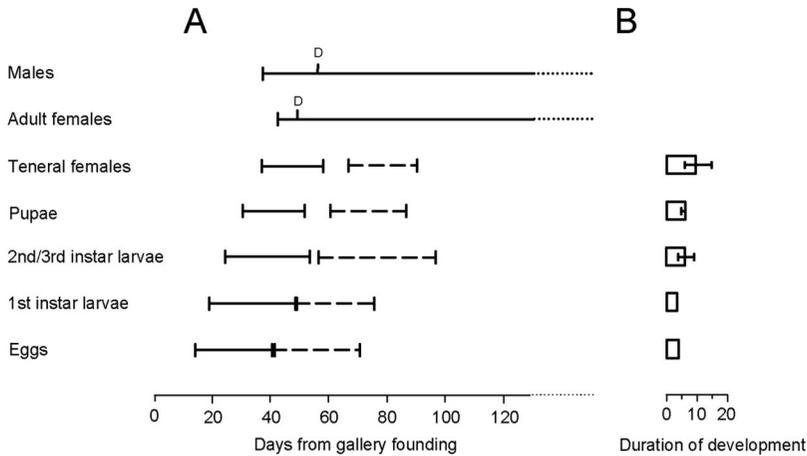
**Fig. 2.** Relationship between the number of *X. saxesenii* foundresses' granddaughters, which is a combined measure of breeding success and productivity of foundresses' daughters and the foundresses' natal gallery productivity in laboratory broods (both rearing media combined; Spearman rank correlation coefficient:  $r_s = 0.637$ ;  $P = 0.026$ ;  $N = 12$ ).



**Fig. 3.** Field and laboratory gallery morphologies of *Xyleborina* species at different times of gallery development. (A) Morphology of an *X. saxesenii* field gallery in beech (*F. sylvatica*) shortly after the hatching of the first larvae. The main tunnel is shown with its entrance to the right and a short side tunnel with the beginning of a small brood chamber. (B) Morphology of an *X. saxesenii* field gallery at a later stage, when all life stages were present. Again, the main tunnel can be seen with its entrance to the right and the species characteristic large brood chamber with a thin fungus layer lining the walls, as well as first- and second/third-instar larvae together with five adult females. (C) Morphology of an *X. saxesenii* laboratory gallery in standard medium at about the same stage as B. A large brood chamber filled with second/third-instar larvae, several pupae, and adult females is visible through the glass of the rearing tubes. (D) Morphology of an *X. affinis* laboratory gallery in standard medium at about the same stage as B and C. Through the glass of the rearing tubes, one can see the species characteristic tunnel system lined by the white fungal layer and inhabited by several second/third-instar larvae and three adult females.

of 2–3, cm they excavated a short side tunnel (2–4 mm long), which was later enlarged into a brood chamber. The brood chamber was always flat, with a height of  $\approx 1$  mm. In the field, this chamber typically expands in the direction of the wood fibers (Fig. 3B and C), which follows the main direction of growth by the wood-penetrating fungal hyphae (visible in the form of the fungus produced blue stain). Growth of *A. sulfurea* Batra was visible in the form of a yellowish layer on the tunnel walls produced after 4–10 d. If this fungus did not start to grow or other fungi invaded the tunnels, females did not produce eggs and continued boring for up to 5 wk (although they usually left the gallery earlier). After feeding on the ambrosia fungi, females laid 5–15 eggs within the short branch tunnel. This occurred within 4–51 d after founding ( $\bar{X} = 18$  d;  $N = 93$  galleries).

Eggs were frequently groomed and moved between main and branch tunnels. Additionally, females constantly browsed the fungal layers lining the walls and maintained their galleries by shuffling boring dust and frass out of the entrance. As soon as larvae hatched (from the ninth day after founding onward), females often blocked the entrance hole of the gallery, remaining motionless for hours. Attempts failed to remove these beetles without harm. When the offspring reached adulthood (from the 27th day after founding onward), they either dispersed by leaving the gallery or stayed and assisted their mother with gallery maintenance and entrance blocking. A second clutch of offspring appeared in 15 of 93 galleries (Fig. 4A), which was usually laid by a daughter, because the foundress often died earlier. Egg developmental periods (5 d) and first-instar larvae (4 d) did not differ



**Fig. 4.** Timing and developmental periods (same scale) of different life stages within the galleries of *X. saxesenii* at  $\approx 25^{\circ}\text{C}$  (data from both rearing media combined). (A) Gallery phenology. The start and end of the bars mark the 25% quartile of the first appearance and the 75% quartile of disappearance of the respective stage. Solid lines mark the first breeding cycle ( $N = 93$  gall.) and dashed lines mark the appearance of a second clutch ( $N = 15$  gall.). D- First dispersal event of females/males. (B) Mean length of developmental periods of the different stages in *X. saxesenii* ( $\bar{X}$ ; whiskers mark the minimum and maximum values), taken from all well-observable galleries ( $N = 5-12$  gall.).

between galleries. From the second/third-instar stage onward, the periods varied between 4 and 17 d (Fig. 4B).

### Discussion

Representatives of the most widespread genera of Xyleborina were raised and behaviorally studied for several generations in the laboratory on media inoculated with the beetles' self-transmitted microorganisms. Slight changes in the composition of the standard rearing medium did not affect the productivity but increased the breeding success of *X. saxesenii* Ratzeburg about five-fold. The less nutritious and water-rich medium (MM) seemed to satisfy the species' demands better and resulted in breeding success similar to that previously found for this species in the field ( $\sim 20\%$ ; Biedermann 2007) and that of *X. germanus* Blandford ( $\approx 29\%$ ; this study). These figures were well below than that of *X. affinis* Eichhoff (80–90%; this study). Previously, *X. affinis* had been reared with a comparable technique, and breeding success ranging between 65 and 90% (Roepert et al. 1980b). Another *Xyleborus* species was found to have breeding success of  $\approx 60\%$  (*X. ferrugineus* Fabricius; Saunders and Knoke 1967). Mean productivities found for *X. saxesenii* (MM: 12 ♀, 1.66 sign; SM: 7 ♀, 0.29 sign) were comparable to its productivity found in the field (10.6 ♀,  $N = 66$  galleries; P.H.W.B., unpublished data). For *X. affinis*, we found much higher productivity (19.4 ♀, 0.96 sign) than previously found with a similar breeding technique (2.8–11.1 ♀; Roepert et al. 1980b). Productivity in the field is unknown for this species.

Both *X. saxesenii* breeding success and productivity were not affected by two time-consuming surface sterilization techniques and refrigeration before introducing females to the medium. The sterilization techniques were useful in eliminating contaminations by

fungal spores from the beetles' body surface (Francke-Grosman 1963, Norris and Chu 1985), but in our study, they had no relevance for the successful establishment of the ambrosia gardens. This result suggests that either *A. sulfurea* suppresses the spread of weed fungi itself or the beetles possess gardening abilities comparable to the weeding and tending of fungiculturing ants (Currie et al. 1999, Currie and Stuart 2001, Mueller et al. 2005). The exact mechanisms by which ambrosia beetles exclude contaminants are unknown. However, the presence of adult *X. saxesenii* is essential for healthy ambrosia gardens and offspring survival (Batra and Michie 1963, Kingsolver and Norris 1977b), and fungus cropping behaviors have been observed (Biedermann 2007) in these beetles. The observation that females can be refrigerated for a few days without any noticeable effects may be of practical use in future studies.

A significant predictor of *X. saxesenii* breeding success was the foundress' origin. Females from laboratory galleries were about twice as successful in producing broods as females originating from field galleries ( $P = 0.053$ ). This difference may have been because of the fact that laboratory females originated from mothers that had already been successful in breeding under laboratory conditions (i.e., high quality females). Breeding success of these laboratory females correlated positively with the number of offspring produced in their parental galleries, with females originating from larger groups having higher fitness than females from smaller groups. This might be because of highly beneficial fungi with copious fruiting bodies (ambrosia cells) and/or sporodochia, resulting in the production of larger broods and in higher rates of fungal transmission from mother to offspring. Genetic quality differences of the matriline or group augmentation effects may also be involved (Aviles 1993, Kokko et al. 2001, Heg et al. 2005, Bilde et al.

2007). Members of larger groups may also have better health and body condition because of higher levels of cooperation in gallery hygiene, fungus tending, and brood care (Biedermann 2007).

All offspring of *Xyleborina* foundresses are very closely related, almost similar to clones, because of haplodiploidy and obligatory sib mating (Peer and Taborsky 2005). It was therefore highly surprising that breeding success and gallery productivity were so variable among females with the same origin under exactly the same substrate conditions (especially in *X. saxesenii*). Apparently, there is variation in female body condition or in the ambrosia fungus cultivar which they transmit. In our sterilization treatment, the reduction of fungal contamination did not increase the beetles' breeding success. We assume, therefore, that variation in brood establishment is related primarily to the transmission of the fungal mutualist. Unlike other *Xyleborina*, *X. saxesenii* females do not carry their ambrosia fungi in the mycetangium but in the gut. At dispersal, the gut physiology changes from a digestive organ to a space for the accumulation of fungal spores (Francke-Grosman 1975). Thus, the first female excrements in the freshly excavated tunnel inoculate the gallery walls with ambrosia fungus. However, this fungus seemed to be frequently lost during dispersal or did not accumulate sufficiently in the gut. By visual inspection of unsuccessful *X. saxesenii* galleries, we could not detect any growth of *A. sulfurea*, which would be essential for the beetles' offspring production, its maturation, and its nutrition in general (French and Roeper 1975, Kingsolver and Norris 1977b). Dispersing females from the same natal gallery sometimes transmit ambrosia fungi from their gut and sometimes they do not (unpublished observations). This fungal spore transmission is presumably influenced by the number of offspring in the natal gallery or more likely by fungus productivity, as seen in the higher breeding success depending on parental gallery productivity (see above).

We found that at least two generations may overlap in *X. saxesenii* galleries. Overlapping generations are regarded as an important step toward the evolution of eusociality (Sherman et al. 1995), which might originate from the ability of daughters to take over brood and fungus care if the mother beetle dies (assured fitness returns; Gadagkar 1990, Queller 1994, Peer and Taborsky 2007). Advanced levels of sociality are expected to exist in *Xyleborina* as this group exhibits high relatedness within groups, because of haplodiploidy and obligatory inbreeding, increasing the potential for indirect fitness benefits from cooperative brood care and fungiculture (Peer and Taborsky 2007). The developmental periods we measured for the different life stages at 25°C (larval stage ≈ 8 d, pupation ≈ 5 d) were exactly the same as those found in *X. ferrugineus* at 28°C (Kingsolver and Norris 1977a). We detected three solely male broods in *X. saxesenii* (brood size 14, 16, and 51 males), indicating that their foundresses were probably not fertilized in their natal galleries. This differs from findings in *X. ferrugineus* (Norris 1972) and *Xyleborus pfeili* Ratze-

burg (Mizuno and Kajimura 2002), where unfertilized foundresses first produced a son and then mated with him to produce daughters.

Galleries and brood chambers were usually aligned to the walls of the glass tubes, which allowed observations within galleries of all three *Xyleborina* studied. Behaviors and development of all life stages inside the gallery and on the fungus gardens were easily observable under a microscope (magnification, ×6.4–40). When autoclavable plastic tubes are used instead of glass tubes, even manipulations of the gallery composition are possible. Fungi and individuals may be added or removed by boring small holes into the tubes and afterward sealing them with hot plastic (Meister 2008), which enables experimental studies on the social systems and the mechanisms of agriculture in these agricultural animals. Our breeding technique could also help to develop control methods for these invasive forest pests, and it might help to expose the secret life of ambrosia beetles to a wider public.

### Acknowledgments

We thank S. Blomqvist and E. Ott for collecting *X. affinis* in the field and starting the first laboratory galleries. Discussions with K. Peer were very helpful to start this study and to improve the laboratory technique. This manuscript emerged from a project developed in a scientific writing course attended by P.H.W.B. and led by M. Fischer at the University of Bern, and it benefited greatly from comments of various students and teachers, T. Turrini, and two anonymous reviewers. Studies in Pineville, LA, were supported by a cooperative agreement with the Southern Research Station, USDA Forest Service.

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*Received 9 November 2008; accepted 28 May 2009.*

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