able, and the only known fossil "mushroom" from the tropics. The record of plants and animals in Dominican amber, as well as the type of tree that produced the amber, clearly indicates that, during the resin production period, the environment was a tropical one (*8*). The present find does not detract from that conclusion. Other fungi that have been described as "Agaricaceae" are either clearly not members of the Agaricales (the four species of *Archagaricon*) or are much more recent (Pleistocene to Miocene) (*9*).

Considering the rarity of fossil agarics, explainable by the difficulties involved in conserving a soft fruiting body composed of thin-walled hyphal cells, the discovery of *C. dominica* is of importance with respect to the evolutionary development of the Basidiomycetes. It has been shown that *Palaeosclerotium* from the Pennsylvanian (*10*) is probably an ascomycete with clamp connections (*11*). However, other Pennsylvanian wood fungi with clamp connections may represent early Basidiomycetes. The oldest confirmed basidiomycete is the Jurassic pore fungus *Phellinites*, a member of the Polyporaceae associated with *Araucaria* trunks in Patagonia (*12*). Considering the "normal" appearance of the fossil mushroom described here, it is likely that the "Agaricaceae" arose much earlier than the early to mid-Tertiary.

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Activation of *ras* Oncogenes Preceding the Onset of Neoplasia

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The identification of *ras* oncogenes in human and animal cancers including precancerous lesions indicates that these genes participate in the early stages of neoplastic development. Yet, these observations do not define the timing of *ras* oncogene activation in the multistep process of carcinogenesis. To ascertain the timing of *ras* oncogene activation, an animal model system was devised that involves the induction of mammary carcinomas in rats exposed at birth to the carcinogen nitrosomethylurea. High-resolution restriction fragment length polymorphism analysis of polymerase chain reaction–amplified *ras* sequences revealed the presence of both H-*ras* and K-*ras* oncogenes in normal mammary glands 2 weeks after carcinogen treatment and at least 2 months before the onset of neoplasia. These *ras* oncogenes can remain latent within the mammary gland until exposure to estrogens, demonstrating that activation of *ras* oncogenes can precede the onset of neoplasia and suggesting that normal physiological proliferative processes such as estrogen-induced mammary gland development may lead to neoplasia if the targeted cells harbor latent *ras* oncogenes.

ACTIVATED *ras* ONCOGENES HAVE been identified in some common forms of human cancer including carcinomas of the lung, colon, and pancreas (*1*). In many instances, these oncogenes are already present in well-defined precancerous lesions (for example, adenomas and myelodysplasias), indicating that they play a role in the early stages of carcinogenesis (*1*). Progression into the more malignant stages of the disease requires additional events often involving either activation of other oncogenes or deletion of growth suppressor genes (*2*).

Animal model systems have been used to elucidate the role of *ras* oncogenes in carcinogenesis (*3, 4*). Indeed, the first indication that *ras* oncogene activation was involved in the early stages of carcinogenesis was provided by study of premalignant mouse skin papillomas (*5*). Moreover, *ras* oncogenes present in tumors induced by carcinogens of known mutagenic specificity often exhibit the type of mutation induced by the initiating carcinogen (*3, 4*). These results have been interpreted as evidence for a direct interaction between the initiating carcinogen and critical *ras* DNA sequences, a proposal that implies that *ras* oncogene activation must be at least concomitant with initiation of neoplasia (*6*). However, since cancer does not develop immediately after carcinogenic exposure, *ras* oncogenes remain latent and require the cooperation of additional factors to trigger neoplastic development. We have investigated the validity of these hypotheses by determining the timing of *ras* oncogene activation and the nature of some of the additional events required for the onset of carcinogenesis.

Carcinogenic exposure during sexual development leads to mammary carcinogenesis in female rats (*7*). Molecular analysis of these tumors has revealed the presence of activated H-*ras* oncogenes, particularly in those mammary carcinomas induced by the direct-acting carcinogen nitrosomethylurea (NMU) (*8–10*). In order to address the temporal relationship between *ras* oncogene activation and the onset of neoplasia, we separated the timing of the carcinogenic insult from sexual development. For this purpose, neonatal (2-day-old) Sprague-Dawley rats were administered a single subcutaneous injection of NMU (50 mg/kg of body weight). These animals developed mammary tumors with an incidence of 80% (35 animals out of 44 treated) and a mean latency of 3.7 months, about 2 months after reaching sexual maturity (Table 1). Most of these tumors were diagnosed as carcinomas (80%), although some benign fibroadenomas (20%) were also observed. Next, we investigated whether these neonatally induced tumors harbored *ras* oncogenes by utilizing a combination of polymerase chain reaction (PCR) amplification (*11*) and allele-specific oligonucleotide hybridization techniques (*12, 13*) to detect *ras* oncogenes activated by G to A transitions, the mutation previously found in all *ras* oncogenes of NMU-induced mammary tumors of rats (*8–10*). Fifteen of the 28 mammary carcinomas (54%) contained G to A mutated H-*ras* oncogenes (Table 1). A similar incidence (three activated oncogenes in seven tumors) was observed in the benign fibroadenomas. In addition to H-*ras* oncogenes, G to A mutated K-*ras* oncogenes were observed in five carcinomas and one fibroadenoma (17% overall incidence). These results indi-

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Table 1. *ras* Oncogenes in rat tumors induced by neonatal exposure to nitrosomethylurea.

Tumor type	Mammary tumors found* (*n*)	Median latency (months)	Tumors with activated oncogenes (*n*)†		
			H-*ras*	K-*ras*	None detected
Carcinoma	28	3.6	15	5	8
Fibroadenoma	7	4.0	3	1	3

*Forty-four female rats were analyzed and 33 of these carried one tumor each in one mammary gland. Two animals had two tumors each. †The activating mutation in each of the *ras* oncogenes was identified as a G to A transition in the second nucleotide of the 12th codon.

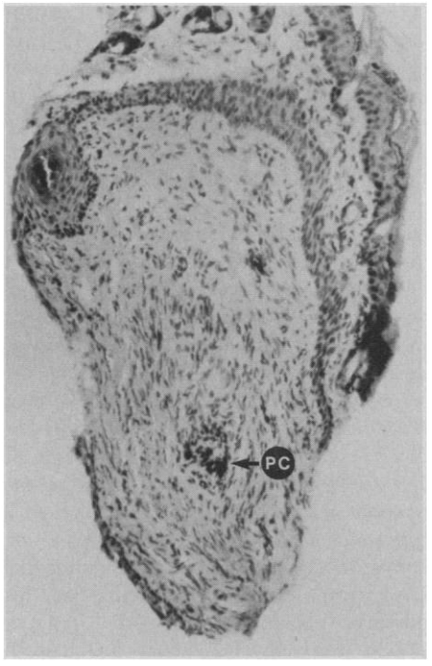

Fig. 1. Mammary tissue (1 to 2 mm in diameter) excised from a 2-week-old rat treated with NMU 2 days after birth. A tangential section stained with hematoxylin and eosin is shown. The cluster of cells located in the lower part of the section (PC) represents the precursor gland cells.

cate that *ras* oncogene activation does not have a significant influence on whether the harboring cell progresses into a benign or a fully malignant tumor. Since mammary fibroadenomas do not develop into malignant carcinomas, it is likely that they arise from a different type of precursor cell that is also susceptible to transformation by resident *ras* oncogenes.

In order to verify that these mutated *ras* genes had transforming activity, DNA was isolated from some of these mammary tumors and tested in gene transfer assays using NIH 3T3 cells as recipients (*14*). DNA from each of six tumors with mutated H-*ras* as identified by PCR yielded foci of transformed NIH 3T3 cells. Transformants were analyzed by Southern (DNA) blot and each transformant revealed the presence of rat H-*ras* oncogene sequences possessing the expected G to A transitions, determined by PCR analysis (*12*). Similarly, DNA isolated from three of the six tumors containing G to A mutated K-*ras* genes transformed NIH 3T3 cells. The lower percentage of transformation observed with DNA isolated from tumors containing K-*ras* oncogenes is likely to be due to the larger size of this gene. No morphologic transformation of NIH 3T3 cells could be observed with DNA from any of the 11 tumors that did not contain G to A activated *ras* oncogenes, confirming that these mammary tumors do not contain *ras* oncogenes activated by mutations other than 12th codon G to A transition. Finally, histopathological analysis of these 35 NMU-induced mammary tumors did not reveal any significant differences between those carcinomas or fibroadenomas carrying activated H-*ras* or K-*ras* oncogenes and those with nonmutated H-*ras* or K-*ras* oncogenes.

Histopathological analysis of mammary glands in rats neonatally exposed to NMU revealed no evidence of neoplastic growth either in the gland precursor cells or in the surrounding stroma and cellular matter until the animals reached sexual maturity (Fig. 1). Therefore, we decided to test whether *ras* oncogenes exist in mammary glands of NMU-treated animals as early as 12 days following carcinogenic exposure. Two weeks of age is the earliest time that individual mammary glands could be surgically removed. At this stage in development, mammary glands are 1 to 2 mm in diameter and contain between 10,000 and 50,000 total cells (depending on the amount of stroma), of which approximately 50 to 100 cells are the precursors of the mature mammary gland. Recent progress in molecular diagnostic techniques has allowed us to identify *ras* oncogenes in single cells even when mixed with up to 10,000 normal cells (*12*).

Mammary glands were excised from seven 14-day-old NMU-treated rats and submitted to PCR amplification of H-*ras* sequences in the presence of specifically mismatched primers that created a diagnostic Xmn I cleavage site only in those samples carrying G to A activated H-*ras* oncogenes (*12, 13*). Eight of the 70 mammary glands tested exhibited the 53-bp Xmn I–cleaved fragment diagnostic of the presence of activated H-*ras* oncogenes (Table 2). Similarly, we amplified K-*ras* sequences using a specifically mismatched primer that in combination with the expected G to A transition created an Hph I recognition site (*13*). In this case, 3 of the 70 mammary glands exhibited the diagnostic DNA fragment indicative of the presence of G to A activated K-*ras* oncogenes. Representative experiments depicting the probe shift assays for H-*ras* and K-*ras* oncogenes are shown in Fig. 2. None of 39 mammary glands isolated from four 2-week-old untreated control rats carried G to A mutations diagnostic of the presence of either H-*ras* or K-*ras* oncogenes. These results demonstrate that *ras* oncogenes exist in cells of morphologically normal mammary glands just 12 days after receiving a carcinogenic insult and well before there is any detectable manifestation of abnormal or neoplastic growth.

We next examined the contribution of the maturation of the mammary gland during sexual development to the carcinogenic process. Twenty-seven 2-day-old female rats were exposed to a single dose of NMU and then treated with the antiestrogen tamoxifen until they were old enough (30 days) to undergo surgical ovariectomy. These animals were monitored for mammary tumor formation for up to 1 year. Only one of them developed a mammary carcinoma (4% incidence), at age 7 months. These results

Table 2. Detection of *ras* oncogenes in rat mammary tissue 2 weeks after neonatal exposure to NMU

Treatment on day 2*	Animal designation	Glands tested† (*n*)	Oncogene positive‡ (*n*)	
			H-*ras*	K-*ras*
None	1	11	0	0
	2	8	0	0
	3	8	0	0
	4	12	0	0
	Total:	39	0	0
NMU	1	11	1	1
	2	11	2	0
	3	7	2	0
	4	7	0	0
	5	12	1	0
	6	11	0	2
	7	11	2	0
	Total:	70	8	3

*NMU was administered subcutaneously to neonatal (2-day-old) rats at a dose of 50 mg per kilogram of body weight. Control animals were injected with buffer. †Mammary glands were surgically removed when the animals were 14 days old. ‡Mutations in the H-*ras* and K-*ras* genes were identified as described in the legend to Fig. 2.

demonstrate that the presence of *ras* oncogenes in the mammary gland of young animals is not sufficient to efficiently trigger neoplastic development. Moreover, the data illustrate the need for *ras* oncogenes to cooperate with physiological processes required for sexual maturation in order to exert their neoplastic properties.

To determine whether estrogen might be one of the elements required for *ras* oncogene–induced carcinogenesis, 12 neonatal female rats were exposed to NMU, treated with tamoxifen, ovariectomized as indicated above, and injected subcutaneously with estrogen (17β-estradiol, 350 μg per kilogram of body weight) 3.5 months after birth. At this time, the treated animals did not show any detectable neoplastic growth (Fig. 3), whereas 50% of the control group, which had been allowed to proceed through sexual maturity after treatment with NMU, had developed palpable tumors. Of the 12 ovariectomized and reconstituted animals, 6 developed mammary tumors with a latency of 2.5 months after initiation of estrogen treatment (Fig. 3). Four of these tumors were diagnosed as malignant carcinomas. Three of these carcinomas, but neither of the two fibroadenomas, contained G to A activated H-*ras* oncogenes, an overall incidence similar to that found in those NMU-exposed animals that were allowed to undergo normal sexual maturation (Table 1). These results demonstrate that H-*ras* oncogenes preexist in a latent form in a subpopulation of mammary gland cells, and that estrogen-induced proliferation or differentiation of such cells can trigger the onset of mammary carcinogenesis.

Our results demonstrate that activation of *ras* oncogenes can precede the onset of neoplasia, and can exist in apparently normal cells for long periods of time without inducing malignant transformation. The latency of *ras* oncogenes in the absence of sexual development might be due to the lack of *ras* gene expression in the NMU-targeted mammary cells, although the ubiquitous presence of *ras* transcripts in mammalian cells makes this interpretation unlikely (*15*). Instead, the absence of cell transformation in these glands might be due to the inability of *ras* oncogenes to induce proliferation of the slowly dividing mammary precursor cells. Only when these cells are induced to actively proliferate by estrogens (either during sexual development or in our reconstitution experiments, Fig. 3) can the resident *ras* oncogenes alter the normal cellular proliferation programs leading to uncontrolled growth. The ability to alter growth might be related to alteration of other genetic components. Depending upon the type of cell that harbors the *ras* oncogene, such uncontrolled growth may result in a benign fibroadenoma or in a fully malignant carcinoma. Cell proliferation alone cannot explain the specificity of tumor development in the model system described here. Cells from a variety of tissues, such as those of the hematopoetic system, are also exposed to the mutagenic effect of NMU and undergo active proliferative programs. It could be argued that, in rats, the mammary gland is more susceptible to NMU-induced *ras* mutagenesis, a premise supported by our inability to detect activated *ras* oncogenes in other normal tissues of these NMU-treated animals (*16*). However, it is likely that additional factors, such as the nature of the specific signal transduction pathways utilized by the proliferating cells, are involved in determining the phenotypic manifestation of the neoplastic properties of *ras* oncogenes.

We propose that *ras* genes may become randomly activated during the life-span of healthy individuals, either by exposure to carcinogenic agents or by sporadic errors during cell replication. These oncogenes would remain silent until certain unscheduled proliferative conditions (such as exposure to growth-promoting agents including cigarette smoking or viruses) or additional genetic alteration would trigger neoplastic development in a fashion similar to that observed in the animal model system described above.

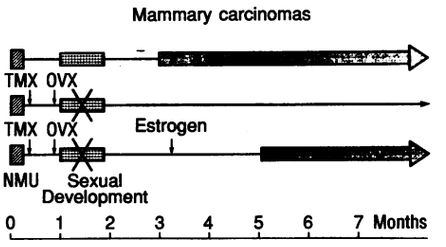

Fig. 3. Induction of mammary carcinomas in Sprague-Dawley rats injected 2 days after birth with a single subcutaneous dose (50 mg per kilogram of body weight) of NMU. Hatched box, NMU treatment; dotted box, sexual development; shaded box, tumor development; TMX, tamoxifen (150 μg per kilogram of body weight, at weekly intervals until ovariectomy); OVX, ovariectomy; and Estrogen, 17β-estradiol (350 μg per kilogram of body weight) at weekly intervals.

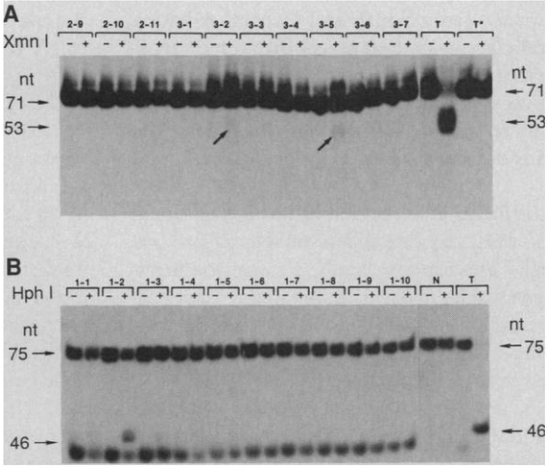

Fig. 2. Detection of *ras* oncogenes activated by G to A transitions in the mammary glands of 14-day-old rats treated with a single dose of NMU 2 days after birth. Glands were excised, washed twice with phosphate-buffered saline, pH 7.5, permeabilized by three cycles of freezing and thawing in distilled water, and submitted to PCR amplification of 71-bp and 75-bp segments of H-*ras* and K-*ras* oncogenes, respectively (*12, 13*). H-*ras* sequences were amplified with a 20-nucleotide (nt) upstream primer (5′-ACCCCTGTAGAAGCGATGAC-3′, positions −15 to +5) and a specifically mismatched (mismatches are underlined) downstream 20-nt primer (5′-AGGGCACTCTTTCGAACGCC-3′, positions +56 to +37). The activating G to A transition in the second nucleotide of the 12th codon (+35) in combination with the specific introduced mismatches creates an Xmn I cleavage site. K-*ras* sequences were similarly amplified with a mismatched upstream primer (5′-AACTTGTGGTAGTTGGAGGTG-3′, positions +14 to +34) and a downstream primer (5′-TCCACAAAGTGATTCTGAATT-3′, positions +89 to +69). The activating G to A transition in the second nucleotide of the 12th codon (+35) in combination with the specific mismatch creates an Hph I cleavage site. Each amplification cycle consisted of denaturation for 1 min at 94°C, annealing for 2 min at 50°C, and initial extension time of 3 min at 72°C, which was increased by 5 s after every cycle. Forty cycles were carried out. Portions of the amplified DNA were incubated in the absence (−) or the presence (+) of 20 units of the appropriate enzyme. The DNA fragments diagnostic of the G to A transition (53 bp for H-*ras* and 45 bp for K-*ras* oncogenes) were detected by the probe-shift assay (*12*), with 5×10^5 dpm of ^{32}P-labeled 20-nt oligonucleotides (5′-CAAGCTTGTGGTGGTGGGCG-3′ for H-*ras* or 5′-CCTTGACGATACAGCTAATT-3′ for K-*ras* sequences) as probes in 30 μl, containing 0.75 M NaCl. DNA was denatured by heating for 10 min at 95°C and quickly cooled to 50°C. Hybridization, at 50°C, was carried out for 2 hours and the resulting hybrids were analyzed by native-gel electrophoresis and autoradiography. Numbers above each pair of lanes indicate animal and individual gland number. (**A**) Detection of H-*ras* oncogene in mammary glands. Mobilities of the wild-type (71 nt) and the diagnostic (53 nt) DNA fragments are indicated. The diagnostic fragment is also indicated with slanted arrows in two glands. T, DNA isolated from an NIH 3T3 cell line transformed by a G to A activated rat H-*ras* oncogene; T*, the same DNA sample amplified with non-mismatched primers. (**B**) Representative results showing the detection of K-*ras* oncogene in mammary glands. Mobilities of the wild-type (75 nt) and diagnostic (46 nt) DNA fragments are indicated by arrows. N, DNA isolated from untreated mammary tissues; T, DNA isolated from a NIH 3T3 cell line transformed by a G to A activated rat K-*ras* oncogene.

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16. Crude cell lysates prepared from liver and spleen were tested as described in (*12*) and (*13*). No G to A mutated H-*ras* or K-*ras* oncogenes could be detected in these samples (R.K. and M.B., unpublished observations).
17. The studies were initiated in the Developmental Oncology Section, BRI-Basic Research Program, NCI-FCRF, Frederick, MD 21701. Supported in part by contract NO1-CO-74101 of the NCI, DHHS to Bionetics Research, Inc., and by grants (to S.S.) from the NCI (1-RO1-CA48943), American Cancer Society (CD-402), and Clayton Foundation. We thank P. Djuricovic for expert help in surgically removing the mammary glands, L. Dunn and T. Rowe for technical assistance, A. Sliski for critical reading of the manuscript, and J. Parton for preparation of the manuscript.

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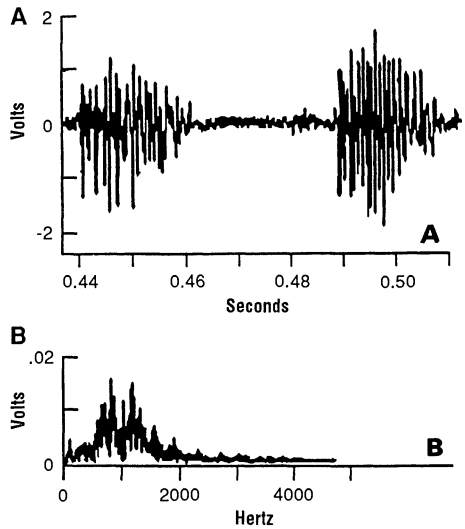

Fig. 1. Waveform of the substrate-borne call produced by a fifth instar *Thisbe irenea* caterpillar. (**A**) An oscillogram trace of two pulses of a typical call. A typical call consists of approximately 16 single pulses per second with an interval between each pulse. (**B**) An amplitude-frequency spectrum showing the dominant frequencies of a typical call.

Enhancement of Symbioses Between Butterfly Caterpillars and Ants by Vibrational Communication

P. J. DeVries

Butterfly caterpillars produce calls that appear to play a role in maintaining symbiotic associations with ants. A survey of butterfly species from South and Central America, North America, Europe, Thailand, and Australia suggests that the ability for caterpillars to call has evolved independently at least three times, and that calling may be ubiquitous among ant-associated species. Because ants use substrate-borne sound in their communication systems, this study points to the possibility that the calls of one insect species have evolved to attract other, unrelated species.

Among butterfly caterpillars, the ability to form symbioses with ants is known only in the families Riodinidae and Lycaenidae (*1, 2*). The nature of these symbioses is that caterpillars provide ants with food secretions in exchange for protection against predators. By use of analogous organs, caterpillars from these two groups may mediate symbioses with ants by producing amino acid and sugar secretions or semiochemicals (*3, 4*). Studies indicate that if caterpillars are found by insect predators without ants, they have no chance of survival (*1, 2*). Thus, there is a premium for any caterpillar species involved in symbioses with ants to maintain a constant cadre of ant guards. In addition to secretory organs, riodinid caterpillars that form symbioses with ants often have a pair of nonsecretory structures termed vibratory papillae (*5*) whose movement has been speculated to convey vibrations to ants (*4, 6*). However, the function of vibratory papillae has never been demonstrated. I report that (i) the vibratory papillae of riodinid caterpillars function as organs for producing acoustic calls, (ii) caterpillars unable to produce calls are experimentally shown to attract fewer ants, and (iii) comparative data suggest that caterpillar calls have evolved at least three times, always as part of symbiotic associations with ants.

Caterpillar calls of a typical ant-associated riodinid butterfly, *Thisbe irenea* (*7*), were studied on Barro Colorado Island, Panama, and surrounding mainland habitats. Caterpillar calls were detected and recorded by using a particle velocity microphone and amplifier connected to a tape recorder, and the recordings were subsequently analyzed for wave form and frequency characteristics (*8*).

Third through fifth instar caterpillars of *Thisbe irenea* all produced low amplitude calls (*9*). The calls were detectable within a 5-cm radius of the caterpillar when the microphone was in contact with the recording substrate (*8*), or if held in direct contact with the caterpillar's body. When the microphone was held 1 mm away from caterpillars or substrate, no calls were detected, indicating that they were entirely substrate-borne. Caterpillars called constantly when walking or when they were prodded by the observer, and rarely when at rest; previous observations have indicated that the vibratory papillae are most frequently used at these times (*4*).

The calls typically consisted of a simple, repeated pulse stridulation (Fig. 1). The mean pulse rate for the calls of 38 individual caterpillars (2-s segment per call) was 16.5 pulses per second (SE, 0.45; maximum, 21.7; minimum, 11.1). Slight variation in number of pulses per second occurred within and between individual caterpillars; this was probably due to the variation in walking speed of each caterpillar or the stress level while being recorded. Analyses of 76 pulses from 20 individual caterpillars showed a mean dominant frequency of 896 Hz and mean high and low frequencies of 1480 and 370 Hz, respectively (*10*).

The ability to call was eliminated in caterpillars that had their vibratory papillae removed (*11*). Except for their loss of call, these caterpillars fed, oscillated their heads, interacted with ants, pupated, and eventually produced adult butterflies. Because new vibratory papillae are produced at each instar, the ability to call returned to all mute caterpillars when they molted to the next instar. Thus, each individual caterpillar became its own control during the course of the study. Those caterpillars with only a single vibratory papilla removed all produced calls (*11*). Vibratory papillae do not develop until the third instar (*4*), and correspondingly, first and second instar caterpillars were all found to be mute. The obvious head movements associated with sound pro-

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