one or DHT but not by E_2; antiandrogen but not antiestrogen suppresses the song of intact males.
14. A. P. Arnold, thesis, Rockefeller University (1974); M. Gurney and M. Konishi, unpublished observations.
15. C. D. Toran-Allerand, *Brain Res.* **106**, 407 (1976).
16. _____, J. L. Gerlach, B. S. McEwen, *Soc. Neurosci. Abstr.* **4**, No. 392 (1978).
17. A. P. Arnold, F. Nottebohm, D. W. Pfaff, *J. Comp. Neurol.* **165**, 487 (1976). The DM may correspond to the brain area labeled ICO in figures 8 and 9 of Arnold *et al.*
18. A. P. Arnold and A. Saltiel, *Science* **205**, 702 (1979).
19. A. P. Arnold, *Soc. Neurosci. Abstr.* **5**, No. 1474 (1979).
20. B. S. McEwen, in *Receptors and Hormone Action*, B. W. O'Malley and L. Birnbaumer, Eds. (Academic Press, New York, 1977), p. 353. Steroid autoradiography does not quantitate hormone receptors per se, but the distribution of hormone-concentrating cells does parallel the content of hormone receptors as assayed biochemically.
21. Supported by NIH grant HD10501 to M.K., NIH training grant GM 0086, the Arthur McCallum Fund, the Spencer Foundation, and the Pew Memorial Trust.

1 October 1979; revised 23 January 1980

Levels of Batrachotoxin and Lack of Sensitivity to Its Action in Poison-Dart Frogs (*Phyllobates*)

Abstract. *Batrachotoxin is present in remarkably high amounts in the skin of* Phyllobates terribilis. *Levels of batrachotoxin tend to be reduced when* P. terribilis *is maintained in captivity, but even after being confined for up to 6 years, these frogs were still at least five times more toxic than other* Phyllobates *species used by natives for poisoning blowgun darts. Batrachotoxin was not detectable in F_1 progeny reared to maturity in captivity. Nerve and muscle preparations from wild-caught frogs and from the nontoxic F_1 frogs were both insensitive to batrachotoxin. The regulatory site controlling sodium-channel activation and permeability appears to have been minimally altered to prevent interaction with batrachotoxin, but is still sensitive to other sodium conductance activators (veratridine, grayanotoxin) to which the frogs are not exposed naturally.*

Batrachotoxin (molecular weight 538), a complex steroidal alkaloid, is one of the most toxic small molecules known (*1*). It owes its toxicity to a selective and virtually irreversible interaction with the voltage-dependent sodium channels in nerve and muscle, leading to a frequency- and concentration-dependent membrane depolarization. Batrachotoxin has been detected in nature in five species of Neotropical frogs, and its seemingly unique occurrence in these frogs is evidence for a monophyletic origin of the redefined genus *Phyllobates* (Dendrobatidae) of lower Central America and northwestern South America (*2*).

Little is known of the biosynthesis of batrachotoxin and its congeners except that they (and nearly 100 other less toxic and structurally simpler dendrobatid alkaloids) (*3*) seem to be produced in granular "poison" glands that are morphologically similar throughout the Dendrobatidae (*4*). Radioactivity was not detected in dendrobatid skin toxins after the administration of labeled cholesterol, mevalonate, and acetate, which seemed likely precursors (*5*). Levels of the batrachotoxin alkaloids differ greatly among the species of *Phyllobates*. In population samples of Panamanian *P. lugubris* and Costa Rican *P. vittatus*, amounts range from undetectable to about 0.8 μg per frog (*6*). Levels are much higher in *P. aurotaenia*, *P. bicolor*, and the recently described *P. terribilis*, which are confined to western Colombia and which are the only frogs known to be used by natives as the source of poison for their blowgun darts (*2*). *Phyllobates terribilis* is by far the most toxic species; the skin of an adult (body length < 50 mm) contains up to 1.9 mg.

The presence of such an extraordinarily toxic substance in the granular skin glands and its secretion onto the skin after attack by a predator would appear to make self-intoxication a possibility. Vesicles in the skin glands presumably serve to store batrachotoxin and prevent its translocation (*4*), but, after release, the toxin should be readily reabsorbed, particularly when the delicate skin is abraded or punctured in the grasp of a predator.

Earlier it was found that whereas nerve and muscle from *P. aurotaenia* (*7*) are virtually insensitive to the action of batrachotoxin, these tissues are highly batrachotoxin-sensitive in *Rana pipiens* (*7*) and another dendrobatid frog, *Dendrobates histrionicus* (*8*). In *P. aurotaenia*, it appeared that the effector site for the toxin associated with voltage-dependent sodium channels was absent or modified, or that the site was desensitized through constant exposure to the toxin. In the present study, we measured the toxicity levels of two adult *P. terribilis* (*9*) reared in captivity and investigated the sensitivity of preparations of their nerve and muscle tissue to the action of batrachotoxin and two other sodium conductance activators, veratridine and grayanotoxin. We also measured skin levels of batrachotoxin in wild-caught *P. terribilis* after they were maintained in captivity for various periods. Finally, we investigated the batrachotoxin and veratridine sensitivity of nerve and muscle from a frog killed 6 years after capture. Our results raise questions concerning the control of toxin production and the levels maintained in the skin of poison-dart frogs.

The average batrachotoxin-homobatrachotoxin content of ten *P. terribilis*, killed at time of capture, was 1140 ± 144 μg (range, 700 to 1900 μg) (*2*, *6*). Eight other individuals from the same locality averaged 540 ± 50 μg (range, 400 to 600 μg) when killed 3 weeks to 1 year after capture—a decrease to 47 percent of the original mean value. After 3 years in captivity, two individuals contained 320 and 480 μg (28 and 42 percent of the value for the freshly caught frogs). Finally, after 6 years in captivity, one *P. terribilis* contained 250 μg, or 22 percent of the original average value. Even with such a reduction, this old captive still contained five times the amount of toxin normally present in its nearest relatives, *P. bicolor* and *P. aurotaenia* (*2*).

Thus, even after 6 years, captive *P. terribilis* still contain relatively large amounts of skin toxins. It seems highly unlikely that batrachotoxin could be stored in the secretory glands for so long; indeed, in this time one might expect complete turnover of the cells that comprise the granular skin glands. We conclude that the frogs continue to synthesize batrachotoxin in captivity, albeit at a reduced rate or at least with less accumulation. Whether lower levels of essential dietary factors (*10*), unnatural crowding, or other laboratory-induced stress—or lack of stress (*11*)—might account for the decrease is unknown.

Two *P. terribilis* reared in captivity were relatively nontoxic, which was quite unexpected considering the apparent continued production of toxins in the parental colony. Batrachotoxin and homobatrachotoxin, if present, were at levels below the limits of positive detection by either color reaction or mouse assay (< 0.05 μg per 100 mg of skin). The extracts were still pharmacologically active (*12*); however, it is uncertain whether this activity was due to the presence of batrachotoxins (at about 0.02 μg per frog) or some other substance. Thus the frogs reared in captivity either did not synthesize the toxins or did not do so at a rate sufficient for measurable amounts to

0036-8075/80/0620-1383$00.50/0

Table 1. Effect of batrachotoxin, veratridine, and grayanotoxin on resting membrane potentials (RMP's) and miniature endplate potentials (MEPP's) of sartorius muscles from a wild-caught *P. terribilis* maintained in captivity for 6 years and from a laboratory-reared F_1 individual. Each value is the mean ± standard error for at least 30 separate fibers sampled at the neuromuscular junction of each of the two muscles; endplate potentials were recorded for at least 2 minutes for each fiber.

Time of exposure (minutes)	Batrachotoxin (5 μM)		Veratridine (20 μM)		Grayanotoxin (50 μM)	
	RMP (mV)	MEPP's per second	RMP (mV)	MEPP's per second	RMP (mV)	MEPP's per second
			Wild-caught P. terribilis			
0	−99.6 ± 2.1	5.3 ± 0.9	−101 ± 2.3	2.7 ± 1.1	−98.8 ± 4.2	4.1 ± 0.8
30	−96.3 ± 1.3*	8.2 ± 1.6*	−92.5 ± 4.6†	14.6 ± 3.1†	−88.2 ± 3.3†	10.2 ± 1.4†
60	−97.9 ± 2.1*	8.3 ± 3.1*	−81.6 ± 6.2†	20.1 ± 3.3†	−84.4 ± 3.1†	14.7 ± 1.8†
			Laboratory-reared F_1 P. terribilis			
0	−98.7 ± 3.8	3.1 ± 1.7	−99.1 ± 3.7	3.4 ± 1.8	−99.4 ± 3.2	2.7 ± 1.0
30	−97.4 ± 4.2*	4.2 ± 2.0*	−90.6 ± 3.6†	10.3 ± 2.2†	−92.6 ± 3.4†	7.5 ± 2.0†
60	−96.8 ± 3.6*	4.6 ± 1.9*	−87.3 ± 4.0†	18.1 ± 4.4†	−86.1 ± 2.2†	13.2 ± 3.6†

*$P > .1$, Student's t-test. †$P < .01$.

accumulate over 2 years. The morphology of the granular (poison) glands of one of the frogs appeared normal (*4*).

Nerve and muscle from the still highly toxic frog maintained in captivity for 6 years and from one of the relatively nontoxic 2-year-old F_1 frogs were insensitive to batrachotoxin (Fig. 1). It appears that the modification of the site at which batrachotoxin prevents inactivation of sodium channels in nerve and muscle preparations is a genetically controlled modification of the sodium-channel macromolecule in *P. terribilis* rather than the result of desensitization caused by constant exposure to the toxin.

It has been proposed that the plant alkaloid veratridine (Liliaceae) acts as a partial agonist at the same site as batrachotoxin. This hypothesis is based primarily on data from studies with cultured cells (*13*). In *P. terribilis*, which appears to be completely insensitive to batrachotoxin, veratridine still partially depolarizes the muscle membrane cells and causes a fivefold increase in miniature endplate potential frequency (Fig. 1 and Table 1). At a concentration of 20 μM, veratridine caused significant depolarization of the junctional and extrajunctional regions of the postsynaptic membrane of sartorius muscle cells from two *P. terribilis* (Fig. 1 and Table 1). Simultaneous recording of the spontaneous miniature endplate potentials also disclosed a significant five- to sixfold increase in miniature endplate potential frequency, indicating a presynaptic action on the nerve terminal (Fig. 1). These effects occurred in muscles kept at 28° to 33°C, and the onset of action began 15 to 20 minutes after the agent was introduced into the experimental chamber. In *P. aurotaenia*, which was virtually insensitive to batrachotoxin, veratridine caused significant depolarization of the membranes of sartorius muscle cells (*7*). However, sensitivity to veratridine was markedly less in muscles of *P. aurotaenia* than in *R. pipiens*. (It appears that muscles of *P. terribilis* are also less sensitive to veratridine than muscles of *R. pipiens*.)

Grayanotoxin, obtained from rhododendron (Ericaceae) leaves, causes a voltage-dependent increase in sodium conductance in the squid giant axon similar to the action of batrachotoxin (*14*). At a concentration of 50 μM, grayanotoxin caused significant membrane depolarization of sartorius muscle cells from both specimens of *P. terribilis* (Fig. 1 and Table 1). The toxin also induced an increase in miniature endplate potential frequency in a manner similar to that of veratridine. A closely related compound, α-dihydrograyanotoxin, caused a 50-mV depolarization in *R. pipiens* sartorius muscle cells at 10 μM (*15*). Thus the

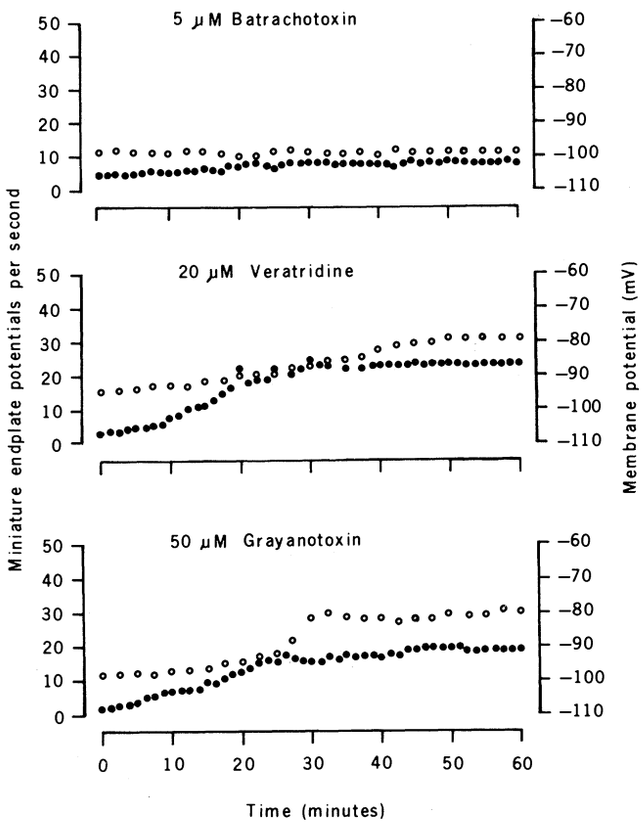

Fig. 1. Miniature endplate potential frequencies (●) and membrane potentials (○) recorded from the two sartorius muscles of a laboratory-reared *P. terribilis*. Each muscle was exposed to batrachotoxin, veratridine, or grayanotoxin for 1 hour. Each data point represents an individual recording from the junctional area of the surface fibers of the muscle. Although the muscles and nerve terminals are insensitive to batrachotoxin, sensitivity to both veratridine and grayanotoxin is still apparent as membrane depolarization and an increase in the frequency of spontaneous transmitter release. (Identical effects were observed with muscles from a wild-caught frog maintained in captivity for 6 years.) The concentration of batrachotoxin (5 μM) is about 100 times greater than that which would cause complete depolarization of *R. pipiens* sartorius muscle within 10 minutes (*7*). The concentration of veratridine (20 μM) is about three times greater than that which would cause complete depolarization of *R. pipiens* sartorius muscle within 10 minutes. The concentration of grayanotoxin (50 μM) is equal to that which would cause 40-mV depolarization of a squid giant axon within 45 minutes (*14*).

muscles of *P. terribilis* appear to be less sensitive to this class of sodium channel activators than the muscles of *R. pipiens*.

Although batrachotoxin, veratridine, and grayanotoxin probably do interact with the same regulatory site that controls activation and inactivation of the sodium channel, this site has been modified in *P. terribilis* (and presumably in the other members of the genus) to prevent interaction with batrachotoxin—although the modification still permits partial interaction with veratridine and grayanotoxin. It seems that the regulatory site is essential to the control of activation and inactivation phenomena and that, from an evolutionary perspective, protection from batrachotoxin was achieved in these frogs with minimal alteration of the site.

JOHN W. DALY
Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205
CHARLES W. MYERS
Department of Herpetology, American Museum of Natural History, New York 10024
JORDAN E. WARNICK
EDSON X. ALBUQUERQUE*
Department of Pharmacology and Experimental Therapeutics, School of Medicine, University of Maryland, Baltimore, Maryland 21201

References and Notes

1. E. X. Albuquerque and J. W. Daly, in *Receptors and Recognition: The Specificity and Action of Animal, Bacterial and Plant Toxins*, P. Cuatrecasas, Ed. (Chapman & Hall, London, 1977), series B, vol. 1, p. 297.
2. C. W. Myers, J. W. Daly, B. Malkin, *Bull. Am. Mus. Nat. Hist.* **161**, 307 (1978).
3. J. W. Daly, G. B. Brown, M. Mensah-Dwumah, C. W. Myers, *Toxicon* **16**, 163 (1978).
4. M. Neuwirth, J. W. Daly, C. W. Myers, L. W. Tice, *Tissue Cell* **11**, 755 (1979).
5. D. F. Johnson and J. W. Daly, *Biochem. Pharmacol.* **20**, 2555 (1971).
6. Levels of batrachotoxin-homobatrachotoxin in the alkaloid fraction of extracts of *Phyllobates* skin were estimated on the basis of toxic reactions in mice to samples from all five species and on chromatographic isolation of large samples from *P. aurotaenia* and *P. terribilis* (2). Time of death of the mice was dose-dependent, occurring from 4 to 14 minutes after subcutaneous injection of 0.3 to 0.1 μg of batrachotoxin (2). Homobatrachotoxin has a toxicity nearly identical to that of batrachotoxin. The presence and approximate amounts of these toxins were confirmed by thin-layer chromatography followed by specific color reaction with *p*-dimethylaminocinnamaldehyde.
7. E. X. Albuquerque, J. E. Warnick, F. M. Sansone, J. W. Daly, *J. Pharmacol. Exp. Ther.* **184**, 315 (1973).
8. E. X. Albuquerque, J. E. Warnick, J. W. Daly, unpublished data.
9. A very limited program for breeding *P. terribilis* was initiated at the American Museum of Natural History in 1976. The source of the breeding colony was a group of 13 frogs obtained in 1973 from the type locality in western Colombia (2). The two laboratory-reared frogs (F_1) used in the present experiments were 21 and 25 months of age (postmetamorphosis), and although they came from different clutches, they may have had shared parentage since the breeding pairs were not sequestered.
10. Wild-caught *P. terribilis* were fed immature crickets dusted with vitamin (Paltone) and calcium powder. Individuals raised in captivity were fed boiled lettuce while they were tadpoles; after metamorphosis they received calcium- and vitamin-dusted *Drosophila* until they were large enough for small crickets.
11. One observation was inconsistent with the tendency toward decreased production or accumulation of skin toxins in captive *P. terribilis*. A specimen caught at the type locality and maintained for 6 years and 4 months was killed 4 days after its body and limbs became grossly bloated because of water retention. Its skin contained 1150 μg of batrachotoxin-homobatrachotoxin, an amount equivalent to the original average value for the wild population and much higher than that of any other frog kept in captivity for more than a few weeks. The possibility should be considered that physiological stress stimulated toxin production in this individual. All other specimens tested for toxicity were apparently in good health when killed.
12. When mice were given subcutaneous injections of an extract equivalent to 100 mg of the skin of a *P. terribilis* individual reared in captivity (total mass of skin, ~400 mg), they experienced locomotor difficulties, gagging, and, after 5 minutes, repeated episodes of clonic convulsions, followed by recovery in 1 to 2 hours. In contrast, an injection equivalent to only 0.4 mg of the skin of a wild-caught *P. terribilis* maintained in captivity for 6 years caused severe convulsions and death within 8 minutes. Thus this individual was over 1000 times more toxic than the frog that was reared in captivity.
13. W. A. Catterall, *J. Biol. Chem.* **252**, 8669 (1977).
14. T. Narahashi and I. Seyama, *J. Physiol. (London)* **242**, 471 (1974).
15. J. G. Starkus and T. Narahashi, *Am. J. Physiol.* **235**, 204 (1978).
16. We thank M. Neuwirth for making electron microscopic comparisons of granular glands from toxic and nontoxic *P. terribilis* (4). Fieldwork was performed in collaboration with the Gorgas Memorial Laboratory, Panama, and the Museo Departamental de Ciencias Naturales, Cali, Colombia, and was partially financed by the Lincoln Ellsworth Fund of the American Museum of Natural History and by a grant from the Camille and Henry Dreyfus Foundation. This research was also supported by PHS grant NS-12063 to E.X.A.
* Reprint requests should be addressed to E.X.A.

6 February 1980; revised 7 April 1980

Genetics and the Origin of a Vector Population: *Aedes aegypti*, a Case Study

Abstract. *Thirty-four population samples representing the worldwide distribution of the mosquito* Aedes aegypti *were analyzed for variation at 19 to 22 enzyme-coding genes. A multivariate discriminant analysis revealed that the genetic differences among populations in six geographic regions and between two subspecies enable one to determine the regional origin of a population. Such studies of population genetics may have quite general applicability in studying vector-borne diseases.*

Geographic variation in the efficiency of disease transmission has been found among populations of a number of insect vectors (*1*, *2*). Thus in certain situations it may be important to know the geographic origin of a vector population. Such information would enable one to determine the potential hazard, for example, of a reinfestation after a successful eradication program. *Aedes aegypti* is the primary vector to humans of yellow fever and dengue fever virsuses, and can also transmit other diseases. We have conducted extensive population genetic studies on the species (*3*). In this report we show that populations of this vector are sufficiently genetically differentiated to allow strong inference of the geographic origin of a population.

We analyzed between 19 and 22 gene

Table 1. The *Aedes aegypti* populations that were sampled, divided into seven regions and subspecies. Subspecific classification follows Mattingly (*8*). Letters in parentheses refer to points on the graph in Fig. 1.

Country	Location	Country	Location
East African formosus *subspecies (A)*		Upper Volta	Kari village
Tanzania	Dar Es Salaam	Upper Volta	Bwombi village
Kenya	Kombeni forest	*Caribbean (D)*	
Kenya	Shimba hills	Jamaica	Montego Bay
Kenya	Kwa Dzivo village*	Puerto Rico	Mayaguez
Kenya	Mombasa	Puerto Rico	San Juan
East African aegypti *subspecies (B)*		*United States (E)*	
Kenya	Kwa Bendegwa*	United States	Indian County, Fla.
Kenya	Majengo*	United States	New Orleans*
Kenya	Mgandini*	*South America (F)*	
West African formosus *subspecies (C)*		Venezuela	Maracay
Nigeria	Enugu City	Venezuela	Caracas
Nigeria	Enugu center	*Asia (G)*	
Nigeria	Ukana	Indonesia	Jakarta
Nigeria	Mamu River forest	Indonesia	Semarang
Nigeria	Egede village	India	Bangalore
Nigeria	Abor village	Taiwan	Kaohsiung
Upper Volta	Bobo-Dioulasso		

*Two samples were obtained, about a year apart (*11*).

0036-8075/80/0620-1385$00.50/0