

TETRODOTOXIN LEVELS IN EGGS OF THE ROUGH-SKIN NEWT, *Taricha granulosa*, ARE CORRELATED WITH FEMALE TOXICITY

CHARLES T. HANIFIN,^{1,*} EDMUND D. BRODIE III,²
and EDMUND D. BRODIE JR.¹

¹*Department of Biology
Utah State University
Logan, Utah 84322-5305, USA*

²*Department of Biology
Indiana University
Bloomington, Indiana 47405-3700, USA*

(Received November 7, 2002; accepted April 13, 2003)

Abstract—We quantified the amount of the neurotoxin tetrodotoxin (TTX) present in females and newly deposited eggs of the rough-skin newt, *Taricha granulosa*, to examine the relationship between the toxicity of an individual female and the toxicity of her eggs. We found high levels of TTX in individual eggs as well as substantial variation among clutches. Variation in the amount of TTX per egg within individual clutches was extremely low. Female skin toxicity was positively correlated with the mean egg toxicity of her clutch. Neither egg volume nor female size was significantly correlated with egg TTX levels. Tetrodotoxin stereoisomer–analog profiles were identical for females and their eggs. The presence of high levels of TTX in individual eggs coupled with the relationship between levels of TTX in female skin and levels of TTX in her eggs suggests that the TTX present in eggs of *T. granulosa* is maternally derived. The lack of correlation between egg size and TTX levels in individual eggs, as well as the low levels of within clutch variation, may indicate that deposition of TTX in eggs of *T. granulosa* is not linked to the deposition of other egg resources (e.g., lipids or other yolk components).

Key Words—Tetrodotoxin, chemical defense, egg toxicity, *Taricha granulosa*, Salamandridae, Caudata.

* To whom correspondence should be addressed. E-mail: chanifin@biology.usu.edu

INTRODUCTION

The rough-skin newt, *Taricha granulosa*, possesses high levels of the neurotoxin tetrodotoxin (TTX) in its skin (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974; Hanifin et al., 1999). Tetrodotoxin serves as a defensive compound in adult newts (Brodie, 1968; Brodie et al., 1974; Daly et al., 1987). Although this toxin is concentrated in the granular glands of the skin (Wakely et al., 1966; Brodie et al., 1974; Tsuruda et al., 2002), it is also present in other tissues, including ovaries, ova, and eggs (Twitty, 1937; Wakely et al., 1966). Tetrodotoxin has also been reported in the eggs or ovaries of other TTX-bearing amphibians, including the frog *Atelopus chiriquiensis* (Pavelka et al., 1977) and the salamander *Notophthalmus viridescens* (Brodie et al., 1974), as well as puffer fish (Goto et al., 1965; Mahmuda et al., 2003). Additionally, Daly et al. (1987) state that TTX is likely present in the eggs of other frog species that possess TTX, but whose eggs and ovarian tissues have not been examined (e.g., other *Atelopus* spp.).

The role of TTX in eggs and reproductive tissues of any species is unclear. In puffer fish, TTX may function as a pheromone (Matsumura, 1995), possibly explaining the high concentrations of TTX found in their ovaries. Tetrodotoxin may serve as an antibacterial or antimycotic agent in amphibian eggs (Brodie et al., 1974; Daly et al., 1987; but see Kim et al., 1975), but the general view is that its primary function is as a chemical defense against egg predation (Twitty, 1966; Daly et al., 1987). Early estimates of egg toxicity in *Taricha* (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974) also indicate that levels of TTX present in newt eggs are probably sufficient for TTX to play a defensive role in those eggs. The defensive role of toxins present in eggs of other taxa has been demonstrated to various degrees. *Bufo* eggs are lethal or unpalatable to known or potential predators of those eggs (Licht, 1967, 1968, 1969; Brodie et al., 1978; Crossland and Alford, 1998). Eggs of the marine gastropod *Dolabella auricularia* possess compounds that increase their unpalatability to possible predators (Pennings et al., 1999), and the importance of pyrrolizidine alkaloids as a defensive mechanism in the eggs of *Utetheisa ornatrix* has been elegantly documented (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000).

In many of the species that produce toxic eggs, the toxins present are either maternally derived (Licht, 1967, 1968; Brodie et al., 1978) or, in cases where the toxin is of exogenous origin, toxin deposition is under active maternal control (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000). Brodie et al. (1974) proposed that the TTX present in eggs of *T. granulosa* originates in the female and is secondarily deposited in eggs. Thus, the toxin present in *Taricha* eggs may be a result of maternal endowment. This maternal endowment of TTX does not require that newts are producing their own TTX; if the ultimate origin of TTX is exogenous, females could still control the deposition of TTX in eggs. Extensive work in a range of taxa (including *Taricha torosa*) has demonstrated that variation in maternally endowed egg resources can have a significant effect on

offspring quality (Kaplan, 1985; Sinervo, 1990, 1999; Gil et al., 1999; Komoroski and Congdon, 2001). The primary focus of this work has been the distribution of energetic resources in eggs and the effect that variation in resource distribution has on offspring quality. However, Komoroski and Congdon (2001) make the point that other maternally allocated egg components (e.g., protective chemicals such as TTX) can affect offspring quality independently of either egg size or offspring size. This type of differential investment of nonenergetic egg resources independent of egg size was demonstrated in zebra finches (Gil et al., 1999) and is well documented in *Utethesia ornatrix* (Dussord et al., 1988; Eisner and Meinwald, 1995; Eisner et al., 2000), but has not been shown in amphibians. If TTX is an important defensive compound in eggs of *T. granulosa*, and the proximate source of egg TTX is maternal (e.g., produced by the mother or, if the ultimate origin of TTX is exogenous, sequestered by the mother), variation in maternal allocation of TTX in eggs could have a significant effect on egg survivorship, and thus on offspring fitness.

Understanding the origin of TTX, as well as its role and the contribution of differential maternal allocation, in the eggs of *T. granulosa* requires detailed information on levels of TTX in individual eggs as well as the females that produced those eggs. Previous work has demonstrated the presence of TTX in eggs (or ova) of *T. granulosa* and *T. torosa* (Mosher et al., 1964; Wakely et al., 1966; Brodie et al., 1974), but these studies could not examine individual female toxicity or directly measure individual egg toxicity. Nor did these studies measure variability in TTX levels within individual clutches or variation between clutches within populations. We measured the TTX toxicity of individual *T. granulosa* eggs with a known "family structure," (i.e., from clutches for which the female toxicity was known). This sampling strategy allowed us to quantify the variance in TTX toxicity within and among clutches of eggs from the same population and to evaluate some of the possible factors important in that variance.

METHODS AND MATERIALS

Sampling. We measured the TTX levels in 68 *Taricha granulosa* eggs and 11 females from a previously studied population (Hanifin et al., 1999, 2002) located in the central Willamette Valley (Benton County, Oregon). Gravid female newts were collected in the spring of 2000 ($N = 10$) and the spring of 2001 ($N = 1$). Females collected in 2000 spontaneously deposited eggs, but were also injected with 10 μ l LHRH (de-Gly¹⁰, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to prolong egg deposition in captivity; the female from the 2001 collection deposited eggs spontaneously. A subset of the eggs deposited by each female were collected within 15 hr of deposition and frozen at -20°C . Eggs from this sample were used for toxin analysis (10 eggs each from 3 clutches, 5 eggs each from 7 clutches in 2000, and 3 eggs from a single clutch in 2001). An additional four gravid females were collected from Benton County in 2001. These females were euthanized and preserved in formalin. Each of these

animals was dissected and all ova counted and used to estimate a typical Benton County clutch size for *T. granulosa*.

Female Morphological and Egg Measurements. Egg diameters were measured to the nearest 0.1 mm, using an ocular micrometer. Because previous reports (Twitty, 1966) and preliminary observations (Hanifin, unpublished data) indicated that TTX is concentrated in the yolk and cytoplasm of the oocyte rather than the gel coat surrounding the egg, we measured the diameter of the oocyte rather than that of the total egg (oocyte + gel coat). Females (except for the animal collected in 2001) were weighed and both total length (TL) and snout-vent length (SVL) were measured. Two circular skin samples (0.2 cm²) were removed from the dorsal mid-body (one on either side of the vertebral column) with a human skin biopsy punch (Acupunch™, Acuderm Inc.). This methodology allows for a high degree of accuracy and repeatability when measuring skin toxicity levels (Hanifin et al., 2002).

Toxin Extraction. Extracts from individual eggs were prepared by grinding a single egg in 100 μ l of extraction buffer (0.1 M acetic acid). This homogenate was mixed thoroughly and heated for 5 min in a boiling water bath. After heating, the homogenate was cooled to 32°C and spun at 13,000 rpm for 20 min in a microcentrifuge. The supernatant was removed and spun again at 13,000 rpm for 20 min in 0.5-ml Millipore centrifuge tubes (Ultrafree-MC, 10,000 NMWL filter units). Aliquots of 20 μ l were used for analysis. Extracts from each skin sample were prepared in the same manner after grinding and extracting in 800 μ l of 0.1 M acetic acid using a 1-ml glass tissue grinder (Kontes). This extraction procedure has been shown to be highly repeatable (Hanifin et al., 1999) and is not a significant source of variance in our analysis.

TTX Assay. The levels of TTX present in our samples were quantified by fluorometric HPLC, modified from Yasumoto and Michishita (1985) and Yotsu et al. (1989). Separation of TTX and TTX analogs was performed on a Develosil RP-Aqueous (0.46 \times 25 cm or 0.46 \times 15 cm, Phenomenex, USA) reverse-phase C30 column with a 25 mM ammonium acetate and 35 mM ammonium heptafluorobutyrate buffer (pH 5.0) containing 1% acetonitrile run at a flow rate of 0.5 ml/min. The eluate was mixed with an aqueous 5 N NaOH solution from another pump (0.9 ml/min) and passed through an ASI 330 postcolumn reactor (1-ml reaction chamber) heated to 150°C to derive analogs to fluoresce. After cooling in a 10-cm water jacket, the fluorescent derivatives were detected by a Rainin Dynamax FL-2 fluoromonitor. The excitation wavelength of the detector was set at 365 nm and the emission wavelength was set at 510 nm. The peak areas were measured and integrated with a Hewlett-Packard 3390a integrator. Peak area concentration curves were calculated using standards prepared from commercial TTX (Sigma).

Statistical Analyses. All analyses were performed using either Statview 4.02, Super Anova (Abacus Concepts), or SAS version 8.1 (SAS Institute). We calculated Pearson product-moment correlations between female skin toxicity and mean egg toxicity of her clutch as well as among the female size estimates (mass,

SVL, TL) and female toxicity. These analyses did not include the female collected in 2001 because morphological data were not available for that female. In addition, we used linear regression to examine the relationship between female skin toxicity and the mean egg toxicity of her clutch. We used a one-way analysis of variance (ANOVA) with clutch as the factor to test for differences in toxicity among clutches as well as differences in egg size among clutches. We used analysis of covariance (ANCOVA) with egg volume as a covariate of TTX to test for the effect of egg volume on individual egg toxicity within and among individual clutches.

RESULTS

The TTX levels of the 11 female newts in our study sample ranged from 0.046 to 0.487 mg TTX/cm² skin (Table 1), with a mean of 0.200 ± 0.042 (SE) mg TTX/cm² skin. Female toxicity was not correlated with any of the female morphological measurements (mass, SVL, or TL) (Table 2). The stereoisomer profile of our animals was also identical to that previously reported (Hanifin et al., 1999, 2002) (Figure 1). Only TTX (peak A) and its apparent equilibrium products (tetrodonic acid (peak D), 4-*epi*TTX (peak B), and *anhydro*TTX (peak C)) were present in the skin of newts from this locality.

Tetrodotoxin also was present, in remarkably high levels, in each of the eggs assayed. The mean egg TTX per clutch ranged from 804 to 2767 ng (Table 1). The stereoisomer profiles of eggs matched the stereoisomer profiles of adults (Figure 1). Tetrodotoxin was present in only trace amounts in isolated gel coats of eggs.

TABLE 1. MASS, STANDARD LENGTH (SVL), TOTAL LENGTH (TL), MEAN EGG TETRODOTOXIN PER CLUTCH, AND FEMALE SKIN TETRODOTOXIN LEVELS FOR *Taricha granulosa*

Female mass (g)	SVL (cm)	TL (cm)	#Eggs	Mean TTX (ng)/egg \pm SE	TTX (mg/cm ² skin)
6.21	6.5	14.0	<i>N</i> = 10	1314.8 \pm 38.3	0.184
9.0	6.6	14.7	<i>N</i> = 10	1642.9 \pm 45.0	0.182
9.0	6.6	15.9	<i>N</i> = 10	820.0 \pm 32.2	0.221
11.44	7.4	16.1	<i>N</i> = 5	858.5 \pm 29.0	0.092
9.33	6.4	13.6	<i>N</i> = 5	2630.6 \pm 65.9	0.190
7.13	6.6	15.1	<i>N</i> = 5	2767.4 \pm 25.1	0.272
10.23	6.7	15.7	<i>N</i> = 5	804.0 \pm 30.1	0.058
10.48	7.1	15.8	<i>N</i> = 5	672.8 \pm 44.1	0.082
10.5	6.8	15.2	<i>N</i> = 5	1823.7 \pm 69.2	0.384
8.18	6.6	13.6	<i>N</i> = 5	967.5 \pm 54.5	0.046
NA	NA	NA	<i>N</i> = 3	2506 \pm 80.1 ^a	0.487 ^a

^a Morphological data were not taken for this female.

TABLE 2. PEARSON PRODUCT-MOMENT CORRELATIONS (ρ) FOR FEMALE MORPHOLOGICAL MEASURES AND FEMALE TOXICITY, MEAN EGG TTX PER CLUTCH, AND EGG VOLUME

	Female	ρ	P	N
Female toxicity	Mass	-0.17	0.65	10
	SVL	-0.28	0.43	10
	TL	-0.006	0.99	10
Mean egg TTX/clutch	Mass	-0.36	0.301	10
	SVL	-0.50	0.140	10
	TL	-0.45	0.190	10
Egg volume	Mass	-0.51	0.136	10
	SVL	-0.26	0.464	10
	TL	-0.10	0.779	10

Mean levels of egg TTX per clutch varied significantly between clutches ($F_{9,55} = 237.7$, $P < 0.001$) (Table 1, Figure 2), and this variation was correlated with female TTX levels ($R = 0.69$, $N = 11$, $P = 0.019$); (Figure 2). No other factors that we measured were important correlates of egg toxicity. None of the female size measures were correlated with mean egg TTX levels per clutch (Table 2). Although egg volume varied significantly among clutches ($F_{9,55} = 3.628$, $P = 0.001$), this variation in egg volume was not correlated with variation of TTX levels among clutches ($F_{1,45} = 0.18$, $P = 0.673$) or within clutches ($F_{9,45} = 0.134$, $P = 0.999$). None of the female size measures were correlated with egg volume (Table 2). Within clutch variation in TTX levels was extremely

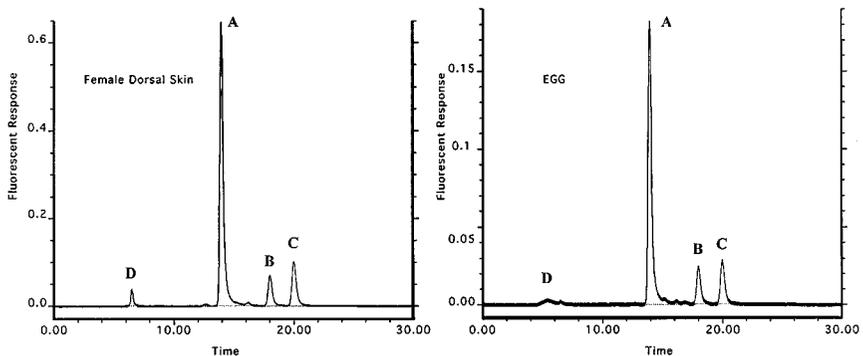


FIG. 1. HPLC-FLD chromatograms of a single typical female *Taricha granulosa* from Benton County, OR (left) and one of her eggs (right) showing the similarity in TTX isomer profiles between female and egg. Note that all analogs present in the female are present in the egg (A = TTX, B = 4-epiTTX, C = anhydroTTX, D = tetrodonic acid).

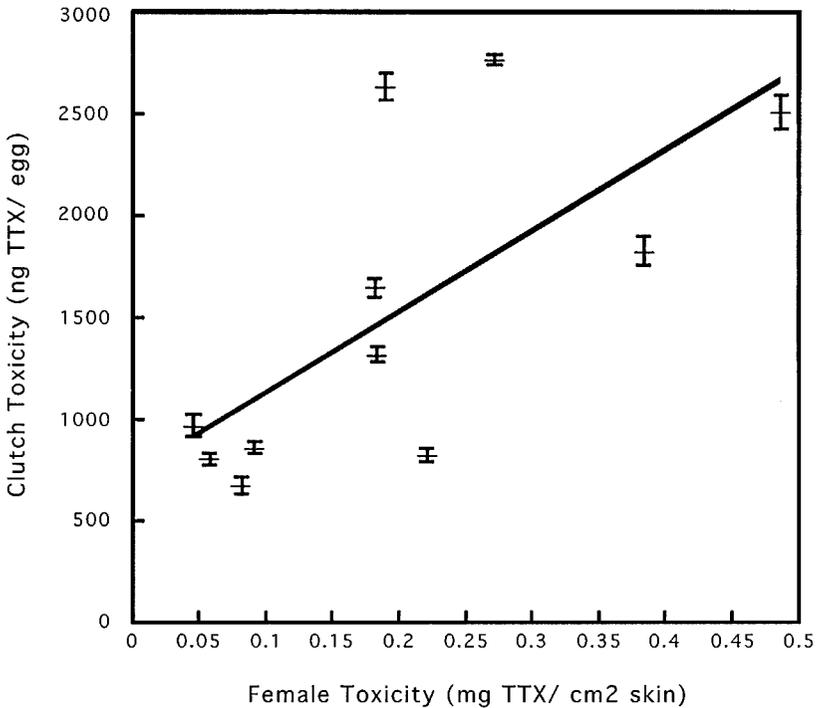


FIG. 2. Graph of egg toxicity versus female toxicity showing a correlation between female toxicity (on the x axis) and mean egg toxicity per clutch (on the y axis). Individual clutches are represented as the mean egg toxicity (light line) with standard error bars. The regression line: Mean Clutch TTX (MCT) = Female TTX*3968 + 735 is significant ($F_{1,9} = 8.128$, $R^2 = 0.48$, $P = 0.019$).

low (Table 1, Figure 2). Mean clutch size (four clutches) was 542 (SD = 110) eggs.

DISCUSSION

Female *Taricha granulosa* with higher levels of TTX in their dorsal skin produced eggs with higher levels of TTX. This correlation between female toxicity and egg toxicity suggests that the TTX present in *T. granulosa* eggs is maternally derived and is allocated by the female. A maternal origin of TTX is further supported by the early developmental stages of the eggs we sampled. In our samples, eggs were frozen within a short time of deposition (no later than 15 hr), and it is unlikely that the TTX present in eggs could be synthesized by developing embryos during that period. Additionally, levels of TTX in *T. torosa* eggs and larvae are

known to decrease as fertilized eggs develop (Twitty, 1937), indicating that new synthesis of TTX does not occur in the eggs while the yolk is being absorbed. Although the TTX present in eggs appears to be a result of maternal allocation, we have no evidence for differential maternal investment of toxin. The correlation between female toxicity and clutch toxicity may indicate that even if females are provisioning their eggs with TTX, they may not be actively controlling the process.

The fact that larger eggs do not possess more TTX than smaller eggs is, however, suggestive that the deposition of TTX in the eggs of *T. granulosa* may be independent of other egg provisioning pathways and, possibly, under maternal control. Other maternally invested resources in *Taricha* are positively correlated with egg size (Kaplan, 1985), while TTX is not, indicating that TTX deposition may not be linked with the deposition of other resources. Likewise, passive diffusion of TTX into eggs should result in larger eggs possessing higher levels of TTX, yet this is not the case in eggs of *T. granulosa*. There is a precedent for active deposition of resources independent of egg size in zebra finches (Gil et al., 1999), but it has not been demonstrated in salamanders. If deposition of TTX in the eggs of *T. granulosa* is independent of other egg provisioning, it could allow selection to act on the process of TTX deposition more easily and also could allow for differential investment of TTX independent of energetic resources.

The amount of TTX present in a single egg, or clutch, of this species is greater than that reported for any other amphibian species in which TTX is present. The average toxicity of a single egg from the most toxic clutch of our study population is 2.767 μg or roughly 14 Mouse Units (M.U.) (Hashimoto, 1979) of TTX per egg. Given that the range of clutch sizes we measured in four females was 400–655 eggs, the total amount of TTX present in a single *T. granulosa* clutch might be as high as 1.8 mg (or approximately 8000 M.U.) of TTX. This level is substantially larger than that reported for *Atelopus chiriquiensis*, which possesses approximately 870 M.U. of TTX per clutch and about 2.5 M.U. of TTX per egg (Pavelka et al., 1977). Estimates from *T. torosa* are more comparable to our results (Mosher et al., 1964; Wakely et al., 1966). Mosher et al. (1964) report that a pooled sample of 100 kg of *T. torosa* eggs (13,000 clutches with a total of $\approx 250,000$ individual eggs) contained 330 mg of TTX or approximately 1.320 μg of TTX per egg, nearly identical to the 1.315 μg of TTX per egg present in our median clutch. Estimates of TTX levels in eggs of other species that possess TTX are less comparable to our results. Brodie et al. (1974) reported that the toxicity of extracts taken from *T. rivularis* ova are roughly similar to those of *T. granulosa* and *T. torosa*, but that extracts of *Notophthalmus viridescens* ova possessed roughly 1/100th the toxicity of *T. granulosa* extracts. This is not surprising given that the skin from adult *N. viridescens* is known to have substantially less toxin than *T. granulosa* (Brodie et al., 1974; Yotsu-Yamashita and Mebs, 2001). The skin TTX levels of females in our study sample were comparable to those previously reported for newts from the Benton County locality. Our mean level of TTX (0.200 ± 0.042 (SE) mg TTX/cm²

skin) is similar to that previously reported (0.169 ± 0.02 (SE) mg TTX/cm² skin from Hanifin et al., 2002).

Levels of TTX in individual eggs are virtually identical within a given clutch. It is impossible to compare within clutch variation in toxicity in *T. granulosa* to that of other toxin bearing amphibians because other studies have not quantified variation within clutches. However, in insects, one species has been tested for within clutch variation in egg toxin levels. In the moth *Utheisa ornatrix*, low levels of variation in within clutch toxicity are an important defensive factor for the eggs (Eisner et al., 2000). Because *T. granulosa* disperses its eggs and *U. ornatrix* deposits a single clutch in one place, the low levels of within clutch variation that we see in *T. granulosa* are not as indicative of a defensive role for TTX in the eggs of *T. granulosa*. Although the similarity of TTX levels within a clutch of *T. granulosa* eggs may not be evidence for a defensive role for TTX in the eggs, it provides additional evidence that toxin deposition in *T. granulosa* eggs may be under some type of maternal control.

The positive correlation between female skin toxicity and egg toxicity, as well as the apparent independence of TTX deposition from yolk deposition, raises the intriguing possibility that adult skin toxicity might experience indirect selection. In adult newts, the efficacy of skin TTX as a defensive compound against vertebrate predators is well understood (Brodie, 1968). If the TTX present in the eggs of *T. granulosa* is also defensive (functioning to deter other vertebrate or invertebrate egg predators) then selection on egg TTX levels would generate correlated selection on maternal toxin levels. Likewise, egg TTX levels could evolve indirectly through the reciprocal pathway. This sort of indirect selection would occur regardless of whether maternal deposition of TTX into eggs is active or passive, and requires only the phenotypic correlation between maternal and egg traits to occur (Lande and Arnold, 1983). Further ecological studies of predation on *T. granulosa* eggs and adult newts are needed to understand the ecology of TTX in the eggs of *T. granulosa* as well as understand the driving forces behind the evolution of egg toxicity in this species.

Acknowledgments—We thank S. Boyes of the Oregon State University Beef Unit for permission and assistance in collecting newts from Soap Creek Ponds. Standards for some TTX analogs as well as technical assistance were provided by M. Yotsu-Yamashita. Jon Hanifin provided assistance with skin sampling. The USU herpetology group provided helpful advice. These studies were supported by the National Science Foundation under grant nos. DEB-9904070 to EDB Jr. and DEB-9903829 to EDB III. Animal care, as well as our tissue sampling protocol, was approved by the Utah State University IACUC panel.

REFERENCES

- BRODIE, E. D., JR. 1968. Investigations on the skin toxin of the adult roughskinned newt, *Taricha granulosa*. *Copeia* 2:307–313.

- BRODIE, E. D., JR., FORMANOWICZ, D. R., JR., and BRODIE, E. D., III. 1978. The development of noxiousness of *Bufo americanus* tadpoles to aquatic insect predators. *Herpetologica* 34:302–306.
- BRODIE, E. D., JR., HENSEL, J. L., JR., and JOHNSON, J. A. 1974. Toxicity of the urodele amphibians *Taricha*, *Notophthalmus*, *Cynops*, and *Paramesotriton* (Salamandridae). *Copeia* 2:506–511.
- CROSSLAND, M. R. and ALFORD, R. A. 1998. Evaluation of the toxicity of eggs, hatchlings and tadpoles of the introduced toad *Bufo marinus* (Anura: Bufonidae) to native Australian aquatic predators. *Aust. J. Ecol.* 23:129–137.
- DALY, J. W., MEYERS, C. W., and WHITTAKER, N. 1987. Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia. *Toxicon* 25:1023–1095.
- DUSSOURD, D. E., UBIK, K., HARVIS, C., RESCH, J., MEINWALD, J., and EISNER, T. 1988. Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix*. *Proc. Natl. Acad. Sci. USA* 85:5992–5996.
- EISNER, T., EISNER, M., ROSSINI, C., IYENGAR, V., ROACH, B., BENEDIKT, E., and MEINWALD, J. 2000. Chemical defense against predation in an insect egg. *Proc. Natl. Acad. Sci. U.S.A.* 97:1634–1639.
- EISNER, T. and MEINWALD, J. 1995. The chemistry of sexual selection. *Proc. Natl. Acad. Sci. USA* 92:50–55.
- GIL, D., GRAVES, J., and HAZON, N. 1999. Male attractiveness and differential testosterone investment in zebra finch eggs. *Science* 286:126–128.
- GOTO, T., KISHI, S., and HIRATA, Y. 1965. Tetrodotoxin. *Tetrahedron* 21:2059–2088.
- HANIFIN, C. T., BRODIE, E. D., III, and BRODIE, E. D., JR. 2002. Tetrodotoxin levels of the rough-skin newt, *Taricha granulosa*, increase in long-term captivity. *Toxicon* 40:1149–1153.
- HANIFIN, C. T., YOTSU-YAMASHITA, M., YASUMOTO, T., BRODIE, E. D., III, and BRODIE, E. D., JR. 1999. Toxicity of dangerous prey: Variation of tetrodotoxin levels within and among populations of the newt *Taricha granulosa*. *J. Chem. Ecol.* 25:2161–2175.
- HASHIMOTO, Y. 1979. Marine Toxins and Other Bioactive Marine Metabolites. Japan Scientific Societies Press, Tokyo.
- KAPLAN, R. H. 1985. Maternal influences on offspring development in the California newt, *Taricha torosa*. *Copeia* 4:1028–1035.
- KIM, Y. H., BROWN, G. B., MOSHER, H. S., and FUHRMAN, F. A. 1975. Tetrodotoxin: Occurrence in atelopid frogs of Costa Rica. *Science* 189:151–152.
- KOMOROSKI, M. J. and CONGDON, J. D. 2001. Scaling of nonpolar lipids with ovum size in the mole salamander, *Ambystoma talpoideum*. *J. Herpetol.* 35:517–521.
- LANDE, R. and ARNOLD, S. J. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210–1226.
- LICHT, L. E. 1967. Death following possible ingestion of toad eggs. *Toxicon* 5:141–142.
- LICHT, L. E. 1968. Unpalatability and toxicity of toad eggs. *Herpetologica* 24:93–98.
- LICHT, L. E. 1969. Palatability of *Rana* and *Hyla* eggs. *Am. Midl. Nat.* 82:296–298.
- MAHMUDA, Y., KENTARO, O., TAKTANI, T., KAWATSU, K., HAMANO, Y., ARAKAWA, O., NOGUCHI, T. 2003. Intra-tissue distribution of tetrodotoxin in two marine puffers *Takifugu vermicularis* and *Chelonodon patoca*. *Toxicon* 41:13–18.
- MATSUMURA, K. 1995. Tetrodotoxin as a pheromone. *Nature* 378:563–564.
- MOSHER, H. S., FUHRMAN, F. A., BUCHWALD, H. D., and FISCHER, H. G. 1964. Tarichatoxin-tetrodotoxin: A potent neurotoxin. *Science* 144:1100–1110.
- PAVELKA, L. A., KIM, Y. H., and MOSHER, H. S. 1977. Tetrodotoxin and tetrodotoxin-like compounds from the eggs of the Costa Rican frog, *Atelopus chiriquiensis*. *Toxicon* 15:135–139.
- PENNINGS, S. C., PAUL, V. J., DUNBAR, D. C., HAMANN, M. T., LUMBANG, W. A., NOVACK, B., and JACOBS, R. S. 1999. Unpalatable compounds in the marine gastropod *Dolabella auricularia*: Distribution and effect of diet. *J. Chem. Ecol.* 25:735–756.

- SINERVO, B. 1990. The evolution of maternal investment in lizards: An experimental and comparative analysis of egg size and its effects on offspring performance. *Evolution* 44:279–294.
- SINERVO, B. 1999. Mechanistic analysis of natural selection and a refinement of Lack's and William's principles. *J. Am. Nat.* 154:s26–s42.
- TSURUDA, K., ARAKAWA, O., KAWATSU, K., HAMANO, Y., TAKATANI, T., and NOGUCHI, T. 2002. Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. *Toxicon* 40:131–136.
- TWITTY, V. C. 1937. Experiments on the phenomenon of paralysis produced by a toxin occurring in *Triturus* embryos. *J. Exp. Zool.* 76:67–104.
- TWITTY, V. C. 1966. *Of Scientists and Salamanders*. Freeman, San Francisco, California.
- WAKELY, J. F., FUHRMAN, G., FUHRMAN, F. A., FISCHER, H. G., and MOSHER, H. S. 1966. The occurrence of tetrodotoxin (tarichatoxin) in Amphibia and the distribution of the toxin in the organs of newts (*Taricha*). *Toxicon* 3: 195–203.
- YASUMOTO, T. and MICHISHITA, T. 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. *Agric. Biol. Chem.* 49:3077–3080.
- YOTSU, M., ENDO, A., and YASUMOTO, T. 1989. An improved tetrodotoxin analyzer. *Agric. Biol. Chem.* 53:893–895.
- YOTSU-YAMASHITA M. and MEBS, D. 2001. The levels of tetrodotoxin and its analogue 6-epitetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*. *Toxicon* 39:1261–1263.