

## SHORTER COMMUNICATIONS

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### Taxonomic Status of the Snake Genera *Conopsis* and *Toluca* (Colubridae)

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The taxonomic history of the colubrid genera *Conopsis* and *Toluca* is complex and has been reviewed by Goyenechea and Flores-Villela (2000). The single character purportedly differentiating them has been called into question by several authors (e.g., Bogert and Oliver, 1945). Some workers recognize just one genus for this group (Bogert and Oliver, 1945; Goyenechea, 1995), whereas others have regarded the two genera as valid (Boulenger, 1894; Dugès, 1896; Duellman, 1961). Taylor and Smith (1942) reviewed these genera and concluded that each was valid. According to these authors, species of *Toluca* have a groove on each posterior maxillary tooth, that is lacking in species of *Conopsis*. In spite of the review by Taylor and Smith (1942), the generic status of *Conopsis* and *Toluca* was questioned by Bogert and Oliver (1945) because the latter did not consider the putative diagnostic character sufficient for recognizing the genus *Toluca*.

In addition to the presence or absence of grooves in the posterior maxillary teeth, another morphological character purportedly differentiating these genera is the condition of the loreal scale (Taylor and Smith, 1942). In *Conopsis*, the loreal scale may be present or fused with the nasal, whereas it is completely absent in *Toluca*. As part of revisionary work on these snakes, we reevaluated these putative, diagnostic features in all recognized taxa of both genera to assess their taxonomic utility, since the only way to allocate specimens to particular species has been on the basis of geographic provenance.

We examined 659 museum specimens, including 199 *Conopsis* and 460 *Toluca* that represented all known taxa (10 species and subspecies) from throughout the geographical range of both genera (both are endemic to Mexico, distributed from Chihuahua to Oaxaca), in order to reevaluate their taxonomic status. The following characters were recorded: snout-vent length (SVL), total length (TL), diameter of the body (DIAM), number of ventral and subcaudal scales, supralabials, infralabials, presence-absence of the nasal, loreal, preocular, postocular, frontal, and genial scales, temporal formula, shape of the hemipenis, and dorsal and ventral color pattern. To determine the presence or absence of tooth grooves, maxillae were dissected on 43 specimens (Appendix 1) representing all recognized species and subspecies of each genus. One maxilla

was dissected in each of six specimens of *Conopsis biserialis* from Guerrero and Morelos; one specimen of *Conopsis nasus labialis* from Chihuahua; seven specimens of *Conopsis nasus nasus* from Distrito Federal, Durango, Hidalgo, Michoacán, Oaxaca and Queretaro; five specimens of *Toluca amphisticha* from Oaxaca; five specimens of *Toluca conica* from Guerrero; six specimens of *Toluca lineata acuta* from Puebla and Hidalgo; four specimens of *Toluca lineata lineata* from Puebla; two specimens of *Toluca lineata varians* from Mexico and Puebla; five specimens of *Toluca lineata wetmorei* from Oaxaca; and two specimens of *Toluca megalodon* from Oaxaca.

All species of *Conopsis* and *Toluca* typically have 12 maxillary teeth, of which the posterior five are enlarged and flanged (10 taxa; Fig. 1). There is no diastema between the smaller anterior teeth and the enlarged posterior teeth. The structure of the flange is the same for all taxa, the posterior ridge of the tooth is extended caudally into a flange or blade, and this leaves a shallow fossa on both the labial and the medial surfaces of the tooth. The maxillary teeth are uniformly conical, becoming larger posteriorly along the maxilla. We found variation in the maxillary teeth among species of both genera regarding the relative size of the teeth, curvature of the fangs, and depth of the flange.

Flanges can be observed on maxillary teeth seven to 12 on all taxa. This condition is common in many aglyphous colubrids. A low, but distinct, flange can be found on *Conopsis biserialis* and *C. n. nasus*. *Conopsis nasus labialis*, *T. l. lineata*, and *T. l. wetmorei* have a more prominent flange, and *T. amphisticha*, *T. conica*, *T. l. acuta*, *T. l. varians*, and *T. megalodon* have the most highly developed flanges.

Loreal scales were present in 31% of the specimens of *Toluca* and 81% of specimens of *Conopsis*. After checking several hundred specimens (the complete list of specimens examined is available upon request to the first author), we attribute this variation to inter-populational differences rather to a feature worthy of generic recognition. In some cases, the loreal scale was present on one side but absent on the other side in the same specimen; similar variation was noted in all the species of both genera (13% in *Conopsis* and 18% in *Toluca*).

Other relatively invariable characters observed in all specimens of both genera include presence of a pair of internasal scales, one preocular and a pair of postocular scales, one rostral, one nasal, one hexagonal frontal scale, and a temporal formula of 1+2. The shape and ornamentation of the hemipenis corresponds to Types A and B of Dowling and Savage (1960), with a subcylindrical shape and reticulated ornamentation with several large spines at the base, respectively. Characters that have been used to define species of *Conopsis* and *Toluca* were found to be variable in all species of both genera. These characters include the number of genial scales, upper and lower labials, the coloration and pattern of spots on both the dorsum and ventrum, and all the morphometric measures we recorded.

Günther (1893) described *C. nasus* for a second time,

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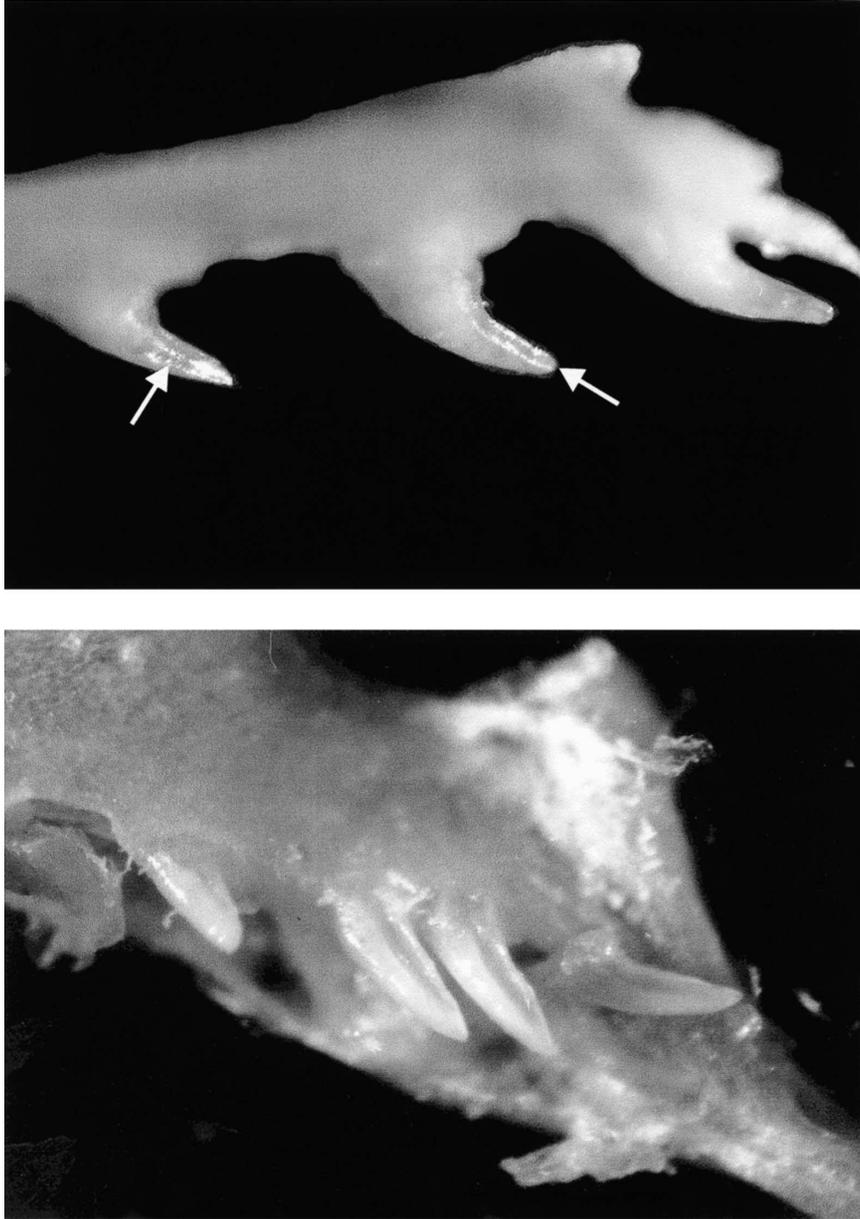


Fig. 1. Maxillae of *Conopsis* and *Toluca* showing the flange in at least one of the rear teeth. Top: *Conopsis nasus* MZFC 617; Bottom: *Toluca lineata wetmorei* MZFC 7568.

as having smooth, equal teeth. However, he also noted, that teeth in "*Conopsis nasus* are not strictly isodont" and observed a "commencement of a groove on large specimens." In their review of the genera *Conopsis* and *Toluca*, Taylor and Smith (1942) argued that Günther (1893) probably confused species of the two genera which at that time were lumped under *Conopsis*, and because of that he saw a faint groove in some individuals. Also, they commented that *Conopsis biserialis* may possess two or three teeth that "may be

very slightly thicker, and a slight depression may be discernible on the outer posterior face."

In contrast to Taylor and Smith (1942), who noted the presence of grooves on the rear teeth of *Toluca*, but described the teeth of *Conopsis* as being smooth, we found that a distinct flange is present in at least the three most posterior maxillary teeth in all of the specimens in both genera, and that the posterior maxillary teeth tend to be enlarged.

Likewise, the condition of the loreal scale is highly

variable within taxa assigned to both genera, and indeed in individual specimens, and cannot be considered a diagnostic character differentiating *Conopsis* from *Toluca*. The diagnostic characters that purportedly separate these genera (sensu Taylor and Smith, 1942) simply do not exist. Therefore, because of the principle of priority, *Conopsis* Günther (1858) must be given priority over *Toluca* Kennicott (in Baird, 1859). All species and subspecies of the former genus *Toluca* should be synonymized under *Conopsis*, and considering that both names have female endings, no changes in spelling of specific or subspecific names are needed.

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#### APPENDIX 1

The maxilla was dissected in the following specimens, museum abbreviations follow Leviton et al. (1985), and Flores-Villela and Hernández-Gómez (1992).

*Conopsis biserialis*: 3603 MZFC GRO, Tetipac, Los Llanos, km 10 carr. Taxco-Tetipac; 3606 MZFC GRO, Ixcateopan de Cuauhtémoc, km 26.5 carr. Taxco-Ixcateopan; 3608 MZFC GRO, Tetipac, Los Llanos, km 10 carr. Taxco-Tetipac; 3612 MZFC GRO, Taxco, Cerro del Huizteco; 3613 MZFC GRO, Pedro Ascencio Alquisiras, 500 m before 3 Cruces de Mamatla; 10167 MZFC MOR, surroundings of Huitzilac, carr. Tres Marias-Huitzilac.

*Conopsis nasus labialis*: 8565 MZFC CHIH, Guachochi; km 28 carr. Creel-La Bufa.

*Conopsis nasus nasus*: 0089 MZFC DF, Iztapalapa, Villa de Guadalupe, Cerro del Guerrero; 0092 MZFC DF, Iztapalapa, Villa de Guadalupe, Cerro del Guerrero; 7026 UTA DGO, Llano Grande; 0617 MZFC HGO, 5 km from Jasso; 2162 MZFC MICH, Patzcuaro Lake; 3344 UTA OAX, Monte Albán; 6235 MZFC QRO, Los Espinos, km 55 carr. Cadereyta-Xilitla.

*Toluca amphisticha*: 12487 UTA OAX, Sierra Mixe, 0.8 km W Totontepec; 12491 UTA OAX, Sierra Mixe, 0.8 km W Totontepec; 14168 UTA OAX, Sierra Mixe, 0.8

km S Totontepec; 14169 UTA OAX, Sierra Mixe, 0.8 km S Totontepec; 14170 UTA OAX, Sierra Mixe, 0.8 km S Totontepec.

*Toluca conica*: 2898 MZFC GRO, Chilpancingo, Omiltemi Salida E del pueblo; 2899 MZFC GRO, Chilpancingo, Omiltemi 2km E-SE; 2900 MZFC GRO, Chilpancingo, Omiltemi on trail to Las Joyas 500 m NW; 2901 MZFC GRO, Chilpancingo, Omiltemi Barranca de Potrerillos; 2902 MZFC GRO, Chilpancingo, Omiltemi 2 km E.

*Toluca lileata acuta*: 3258, 3258-3, 3258-4, 3258-6, 3258-7 MZFC PUE, Chapulco, 4 km E.

*Toluca lileata acuta* × *Toluca lineata lineata*: 0840 MZFC HGO, Tejocotal approx 500 m NE of town.

*Toluca lineata lineata*: 3216 MZFC PUE, town of Amozoc; 3217-18 MZFC PUE, Chignahuapan, Puente rojo 0.5-1 km W; 3534 MZFC PUE, Chignahuapan, Chignahuapan 10 km S.

*Toluca lineata varians*: 7108 MZFC MEX, Atlacomulco km 21 carr. Toluca-Atlacomulco; 5739 MZFC PUE, Tehuacan, 8 km E Chapulco.

*Toluca lineata wetmorei*: 11453-54 MZFC OAX, Cerro de Yucunino; 11455-57 MZFC OAX, Llano de Guadalupe.

*Toluca megalodon*: 6557 MZFC OAX, Sierra de Juárez, km 148 carr. 185 Oaxaca-Tuxtepec; 8301 MZFC OAX, Sierra de Juárez, La Cumbre carr. Oaxaca-Tuxtepec.

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### Recovery of Garter Snakes (*Thamnophis sirtalis*) from the Effects of Tetrodotoxin

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The arms race analogy is a popular view of evolutionary interactions between predators and prey but one whose generality is questionable because of the paucity of empirical studies in natural systems. Predators and prey are expected to experience asymmetrical selection from ecological interactions, leading some authors to question whether predators are under direct selection to respond to evolutionary advances by prey. The consequences of interactions are generally less severe for predators than for prey (the "lifer-dinner principle"; Dawkins and Krebs, 1979) and even severe consequences may not be predictable ("dodging the bullet"; Brodie and Brodie, 1999a). Consequently, evolutionary arms races between predators and prey are most likely to occur when prey are dangerous and, therefore, exert strong selection on predators (Brodie and Brodie, 1999a).

Strong selection by prey on predators is expected when prey are toxic or otherwise dangerous (Brodie and Brodie, 1999a). For toxic prey, selection may result

from the immediate effects of toxins (i.e., injury or death) or from indirect effects that result from the action of the toxin such as temporary immobility, alterations to physiology or metabolism, or reduced organismal performance. Because evolutionary response of predators to prey might result in any predator adaptation that ameliorates the fitness deficits associated with prey toxins, predator resistance might include behavioral avoidance of toxic prey, reduced susceptibility to a toxin directly, or reduced duration of the effects of a toxin, or some combination thereof.

From a microevolutionary perspective, one of the best documented predator-prey systems includes the newt *Taricha granulosa* and its predator, the garter snake *Thamnophis sirtalis* in the Pacific Northwest of North America (Brodie and Brodie, 1990, 1991, 1999b,a). Newts of the genus *Taricha* possess tetrodotoxin (TTX; Mosher et al., 1964; Wakely et al., 1966; Brodie, 1968; Brodie et al., 1974; Daly et al., 1987), an extremely potent neurotoxin that acts as a Na<sup>+</sup> channel blocker. Although all three species of the genus *Taricha* possess this toxin, *Taricha granulosa* is many times more toxic than any other species. The only predator known to forage on newts and resist the effects of this toxin is *Thamnophis sirtalis*.

Within populations, resistance to TTX varies among neonate snakes and has a heritable basis (Brodie and Brodie, 1990). Resistance is not affected by either short-term or long-term exposure to TTX (Brodie and Brodie, 1990; Ridenhour et al., 1999), hence, environmental effects are unlikely to explain familial differences. Among populations, the levels of both newt toxicity and garter snake resistance are variable but roughly matched (Brodie and Brodie, 1990, 1991, 1999a), suggesting a geographic mosaic of coevolutionary outcomes (Thompson, 1994, 1999a,b). Garter snake populations allopatric with newts are not resistant to TTX, whereas sympatric populations are resistant (Brodie and Brodie, 1990). Island populations of newts from British Columbia lack TTX (Hanifin et al., 1999), and garter snakes from these populations are nonresistant (Brodie and Brodie, 1991). Other garter snakes that coexist with *Taricha* are susceptible to TTX (Brodie, 1968; Brodie and Brodie, 1990; Motychak et al., 1999), supporting the view that resistance to TTX is an adaptation by a predator to its toxic prey.

Past studies of TTX resistance have used a bioassay based on locomotor performance to assess variation (Brodie and Brodie, 1990, 1991, 1999b; Ridenhour et al., 1999). The bioassay examines an individual snake's reduction in crawl speed 30 min after an injection of TTX. The bioassay is ecologically relevant because reduced locomotor performance is expected to impair the ability of snakes to escape predators or thermoregulate. The length of time that a snake is impaired from a given dose of TTX is unknown and may further contribute to the selective scenario in the interaction between newts and snakes. In this paper, we explore the time to recovery for individual snakes from a population resistant to TTX. We further examine the correlation between resistance as measured by reduction in locomotor performance after 30 min and the relative recovery at longer intervals.

The subjects for this experiment were neonate *T. sirtalis*, born during the summer of 2000 in captivity to wild-caught females from Benton County, Oregon.

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Gravid females were collected in June 2000, housed individually in 25 × 50 × 30 cm terraria, and fed fish or newts once a week until parturition. Fifty-five neonates from four litters were used in the experiment.

The effects of exposure to TTX were tested with a whole organism performance bioassay developed by Brodie and Brodie (1990). Each individual snake was raced at 26°C (± 1°) on a 2-m linear racetrack, lined with artificial turf, and equipped with infrared sensors at 0.5-m intervals to electronically time sprint speed. The maximum 0.5-m speed in a given trial was taken as the sprint speed score. Individuals were first measured for sprint speed on the third day of life. Trials were repeated after four hours, and the mean of these two trials was taken as an individual's "Baseline Speed." Repeatability of this assay of Baseline Speed in previous studies of *T. sirtalis* from Benton County and other localities ranges from 0.5–0.7 (Brodie and Brodie, 1990, 1999b; unpubl. data). Twenty-four hours after the last sprint test, neonates were given intraperitoneal injections of known quantities of TTX. Thirty minutes after injection, snakes were tested again to obtain a measure of "Postinjection Speed." "Resistance" was scored as the percentage of an individual's baseline speed crawled after injection (Postinjection Speed/Baseline Speed). Individuals that are greatly impaired by TTX crawl only a small proportion of their normal speed, whereas those that are unaffected by an injection of TTX crawl 100% of their baseline speed. Control injections of physiological saline have no effect on snake performance (Brodie and Brodie, 1990), and TTX resistance is not affected by repeated exposure (Brodie and Brodie, 1990; Ridenhour et al., 1999). Compared to ingested TTX, interperitoneal injections of TTX have the same qualitative effect on *T. sirtalis* (Brodie, 1968) but require approximately 40× less TTX for a comparable quantitative reduction in locomotor performance (Williams et al., pers. comm.). The sprint speed bioassay of resistance is highly repeatable (0.5–0.7; Brodie and Brodie 1990, 1999b; unpubl. data) and enables us to assess individual differences in susceptibility to TTX.

We examined the effects of two different doses of TTX (Sankyo, Lot B19481) mixed in 0.1 ml of amphibian Ringer solution (Carolina Biological Supply Co.). All individuals were injected from the same stock solution of a given dose. The doses were selected because they were known to reduce individual crawl speed in snakes from the Benton County, Oregon population to an average of approximately 54% (at 0.0001 mg; the 50% dose) and 17% (at 0.0005 mg) of Baseline Speed 30 min after injection (Brodie and Brodie, 1999a). Within population variation in neonate mass is uncorrelated with resistance in *T. sirtalis* populations that are resistant to TTX (Brodie and Brodie, 1990, 1999b); thus, no attempt to adjust doses to size differences among individuals was made. At the 0.0005 mg dose, subjects were retested at 90 min, 180 min and 360 min after injection, whereas individuals were only retested at 90 min at the 0.0001 mg dose. At this lower dose, virtually every individual had fully recovered after 90 min.

To determine whether individuals with higher resistance also recovered from the effects of TTX more quickly, nonparametric correlations (Spearman's rho,  $\rho_s$ ) were estimated between the percent base crawl

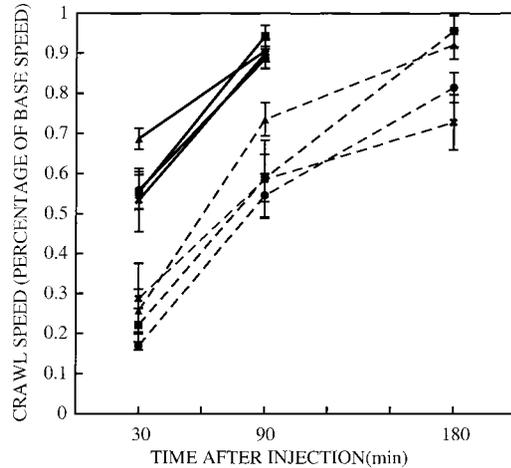


FIG. 1. Percent Baseline Speed as a function of time after injection of TTX. Responses to 0.0001 mg are indicated by solid lines, and responses to 0.0005 mg are indicated by dashed lines. Each line represents a family mean (± SE), with families represented by the same symbol at each dose.

speed at 30 min (resistance) and the percent baseline speed at 90 min and 180 min. Because of potential autocorrelations between these measures (i.e., low values of % baseline speed at later times could not occur in individuals with high initial resistance), six individuals with extremely high initial resistance (> mean + 2 SD) to the 0.0005 dose were removed from all analyses. Removal of these individuals from the analyses reduced the skew in the distribution of resistance, minimizing potential autocorrelations. This censure did not alter the qualitative results of the analyses.

Neonate *T. sirtalis* in this sample ( $N = 55$ ) were reduced to an average of  $57.7 \pm 2.6$  (SE)% and  $21.0 \pm 1.9\%$  of their Baseline Speeds at 0.0001 and 0.0005 mg of TTX, respectively. At the 50% dose the population average had recovered almost fully ( $90.1 \pm 1.2\%$  of Baseline Speed) 90 min after injection (Fig. 1), and every individual had recovered to 69% of Baseline Speed or greater. At the higher dose recovery was slower, with the population average reaching only  $59.8 \pm 3.2\%$  after 90 min. After 180 min, individuals had recovered to an average of  $84.4 \pm 1.9\%$ , and only two individuals had not recovered to at least 60% of Baseline Speed. The subset of 16 individuals that had not recovered to at least 80% at 180 min was tested again at 360 min, and had reached an average of  $79.5 \pm 2.2\%$  at this time. Only two individuals of this subset had not recovered to at least 71% after 360 min.

At the higher dose, resistance at 30 min was positively correlated with both percent Baseline Speed at 90 min ( $\rho_s = 0.470$ ,  $P < 0.0003$ ) and 180 min ( $\rho_s = 0.389$ ,  $P < 0.0033$ ). Resistance and recovery at 90 min were not related at the lower dose ( $\rho_s = 0.074$ , ns).

*Thamnophis sirtalis* recover from the effects of injected TTX surprisingly quickly, but time to recovery depends on the dose and initial effect (Fig. 1). At the 50% dose, virtually all individuals were crawling within 10–15% of full speed after only 90 min (recovery may have been even quicker, but we did not assay

recovery at earlier times). Even at higher doses that virtually immobilized some individuals, the majority of the sample was near full performance after 180 min. Only two individuals had not reached 80% of full capacity after 6 h. The relationship between these recovery times and the actual impairment experienced by snakes that ingest newts is unclear for several reasons. The effects of ingested TTX are quantitatively different than injected TTX (Williams et al., in press). Furthermore, substantial variation exists among individual newts in toxin level (Hanifin et al., 1999) and among individual snakes in resistance (Brodie and Brodie, 1990, 1999a); thus, duration of effect of TTX is likely to be a function of individual predators and prey. We have observed adult *T. sirtalis* to be noticeably impaired for up to seven hours after eating an adult newt in both field and laboratory situations (Brodie and Brodie, 1990).

Impaired locomotor function may represent several selective consequences (Brodie and Brodie, 1990). After eating a meal, snakes must thermoregulate to aid in digestion (Stevenson et al., 1985); hence, a snake that has eaten a newt is likely to be both exposed and impaired immediately following a predation event. As a result, the effects of TTX toxicity may reduce survival in two distinct ways. First, an exposed and debilitated snake is at higher risk of predation. The greater and longer the snake is impaired, the greater the risk. Second, a snake that becomes immobilized for even a short period of time may be unable to avoid lethal temperatures as the thermal environment changes (Peterson, 1987). The longer the duration of impairment from TTX, the greater is the risk from either source of selection.

Time to recovery is related to resistance, as previously measured by reduction in locomotor performance but only at higher doses. The same relationship may hold at lower doses, but the rate of recovery was sufficiently rapid in our experiment that only minimal variation in recovery at 90 min was present at the dose resulting in a 50% loss of ability. The relationship between time to recovery and resistance suggests that individual *T. sirtalis* have a general ability to withstand the effects of TTX in both the degree of initial impairment and duration of impairment. Because the mechanism of TTX resistance in *T. sirtalis* is not yet known, it is unclear whether the physiological bases of resistance and recovery are related. Likewise, it is unclear whether the ecological costs that covary with resistance (Brodie and Brodie, 1999b) also covary with recovery time.

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### Microhabitat Use and Orientation to Water Flow Direction by Tadpoles of the Leptodactylid Frog *Thoropa miliaris* in Southeastern Brazil

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Frog larvae develop in a wide array of microhabitats including permanent and nonpermanent ponds, rivers, lakes, tank bromeliads, and other water reservoirs (Duellman and Trueb, 1994). A unique form of development and microhabitat specialization occurs in tadpoles of leptodactylid frogs of the genus *Thoropa*: tadpoles live in the film of water on rock surfaces at the wet borders of waterfalls in rain-forest areas, and in rocky fields of mountain ranges of southeastern Brazil (Bokermann, 1965; Caramaschi and Sazima 1984; Cocroft and Heyer, 1988). The tadpoles of this genus have an elongated, dorsoventrally compressed body, reduced fins, an expanded and flattened abdomen with an adherent ventral disk, and a long muscular tail, which, together with the labium, are used for movement and adhesion to the substrate (Bokermann, 1965; Wassersug and Heyer, 1983).

*Thoropa miliaris* (Spix, 1824) occurs near freshwater bodies along the Atlantic rain forest of the states of Rio de Janeiro, São Paulo, Minas Gerais, and Espírito Santo in southeastern Brazil (Cocroft and Heyer, 1988) and also on rocky marine shores (Abe and Bicudo, 1991). Information available on microhabitat use by tadpoles of this species is restricted to general notes reporting the use of a very shallow film of current water on rock surfaces at the side of waterfalls with varying slopes, including vertical. These reports suggest that the tadpoles tend to remain with the head oriented against the water flow when adhered to the rock surface (Bokermann, 1965; Abe and Bicudo, 1991). However, there is no quantitative description of microhabitat use by this species. In this study, we analyze microhabitat use by *T. miliaris* tadpoles to address the following questions. Do tadpoles orient randomly or selectively in relation to water flow? Do tadpoles tend to remain at the shallowest sites or use the available film of water indiscriminately? Do tadpoles occupy some slopes available on the waterfall surface more than others? Does water temperature where tadpoles occur differ from that of the main drainage of the waterfalls nearby?

We studied microhabitat use by *T. miliaris* tadpoles along the sides of a waterfall with wet granite substrate. The waterfall is located in the Barra Grande River at a place locally called Mãe D'água Dam (23°10'92.3"S, 44°12'04.4"W) at Ilha Grande, Rio de Janeiro State, southeastern Brazil, at an altitude of approximately 90 m above sea level. The study was carried out in November and December 1998.

Study sites were delineated using a 1 × 1 m wooden frame internally subdivided with cotton strings in 25 subplots of 20 × 20 cm. These subplots were considered sampling units. We systematically sampled all the wet rocky area at both sides of the waterfall. We considered this area the small drainage compared to the main drainage of the waterfall. At each subplot we checked for the presence of tadpoles of *T. miliaris*. Although the water volume and current at the main drainage prevented us using the same methodology, we also checked along the main drainage for the presence of tadpoles of *T. miliaris*. Whenever a tadpole was found within a subplot, we placed a toothpick to indicate its original position and orientation relative to the water flow direction. We included only tadpoles that had not been disturbed by our presence. Because tadpoles of *T. miliaris* are found in very shallow water, sometimes it was not possible to determine visually the direction of the water flow. In such cases, we dropped a pinch of powder on the water beside the tadpole to identify the direction of the water flow. We defined the direction of water flow as zero degree (0°) on a hypothetical circle, and we recorded to the nearest 1° the tadpole's head orientation, relative to the water flow direction, using a protractor. To randomize the possible influence of sunlight on tadpole compass orientation, we recorded observations throughout the day, from early morning to late afternoon. We also recorded, at the center of each 20 × 20 cm subplot, the slope of the rock (using a clinometer, to the nearest 1°) and the depth of the water film. For depth measurements, we put a dried toothpick perpendicularly to the wet rock surface and removed it immediately after it had touched the rock surface; we then measured the length of the wet column on the toothpick using a digital caliper, to the nearest 0.1 mm. The water depth in cells containing tadpoles was measured immediately adjacent to the original position of the tadpole. We estimated tadpole density by counting the number of tadpoles found in each square meter of wet rock surface.

The relationship between tadpole body orientation and the direction of water flow was tested using statistics for circular distributions. A mean vector direction (the mean vector with length "r") expresses the dispersion of points around the mean angle ("a") of orientations around the circle (Batschelet, 1965). The mean vector length (0 < r < 1) varies according to the concentration of data around the mean angle, where r = 1 indicates that all points are concentrated in the same direction (Batschelet, 1965; Gandolfi and Rocha, 1998). We also tested for differences between the depth of the water where tadpoles occurred and the depth of water in subplots where no tadpole occurred, using one-way analysis of variance (ANOVA; Zar, 1999). To evaluate whether tadpoles of *T. miliaris* preferred particular substrate slopes, we compared the frequency distribution of slopes used by tadpoles with the frequency distribution of slopes from all subplots. For this comparison, we used the Kolmogorov-Smirnov test for two groups (Zar, 1999).

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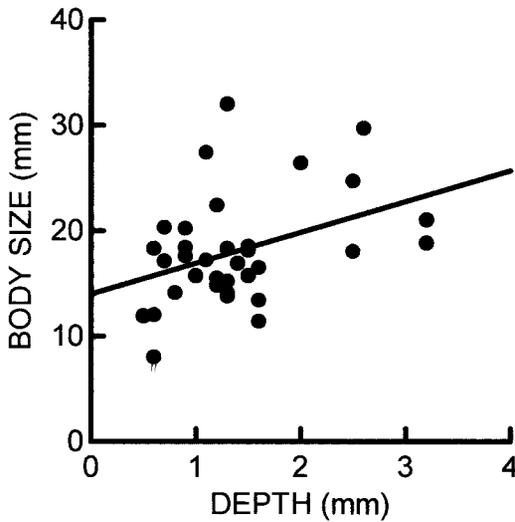


FIG. 1. Relationship between tadpole body size and water depth at the place they were found at the waterfall in Atlantic rain forest of Ilha Grande, Rio de Janeiro State in Brazil ( $R^2 = 0.15$ ;  $F_{1,32} = 5.755$ ;  $P = 0.02$ ).

In both cases, the slopes found were grouped in 18 5°-classes, from 0° to 90°.

A paired *t*-test was used to compare the water temperature between the subplots where tadpoles were found and the main drainage of the waterfall. We tested for normality of data, and nonparametric statistics were used when assumptions of normality could not be met.

We sampled 43 m<sup>2</sup> of rock surface at both sides of the waterfall. Of these, only 17.4 m<sup>2</sup> were considered to be in the small drainage with shallow water. Tadpole size (body and tail length) varied from 8.0–32.0 mm ( $\bar{x} \pm SD = 18.0 \pm 5.2$ ;  $N = 36$ ). The density of tadpoles of *T. miliaris* during the period of the study was 2.01 individuals/m<sup>2</sup> of small drainage. We did not find any tadpoles of *T. miliaris* in the main drainage; tadpoles occurred exclusively in small drainages with shallow film of water. Depth of water in the small drainages was  $1.86 \pm 1.31$  mm (range 0.4–17.4), whereas depth of the film of water in the subplots where tadpoles occurred was  $1.39 \pm 0.7$  mm (range 0.5–3.2) and differed significantly from that found for the small drainage in general (ANOVA;  $F_{1,449} = 4.666$ ;  $P = 0.031$ ). There was a significant relationship between tadpole body size and water depth where they were found ( $R^2 = 0.15$ ;  $F_{1,32} = 5.755$ ;  $P = 0.02$ ). Smaller tadpoles generally were found in shallower water than larger ones (Fig. 1).

Mean water temperature at the small drainages where the tadpoles occurred ( $28.1 \pm 2.19^\circ\text{C}$ ) was significantly higher than that of the water adjacent to the main drainage ( $21.0 \pm 0.23^\circ\text{C}$ ;  $t_{12} = 11.104$ ;  $P < 0.001$ ). Bokermann (1965) first reported that *T. miliaris* tadpoles are found in relatively warm water. Some amphibian larvae (e.g., *Rana sylvatica*, Herreid and Kinney, 1967; *Bufo terrestris*, Noland and Ultsch, 1981; and *Taricha rivularis*, Licht and Brown, 1967) show preferred temperatures when subjected to a thermal gradient. The shallow film of water where *T. miliaris* tadpoles remain heats more easily than water in

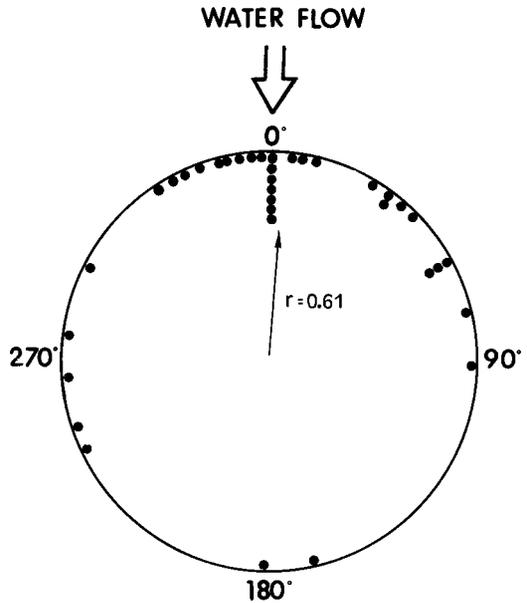


FIG. 2. Tadpole orientation in relation to the water flow. Each black dot represents the orientation of an individual tadpole relative to the direction of water flow in the shallow film of water where they occurred in the waterfall. Arrow indicates the mean angle (3°). The length of the mean vector  $r = 0.61$ . The Rayleigh test ( $z = 13.536$ ;  $P < 0.001$ ).

the main drainage, because the rock surface is exposed to direct sunlight during almost all the day, and the water is shallow and slow moving. Whether the larval behavior of remaining in the warmest water in the waterfalls is a result of their microhabitat selection for shallow (and, thus, warmer water), or a physiological preference for warmer water, deserves further study.

The distribution of slopes in the subplots where tadpoles were found did not differ statistically from the distribution of the slopes of all rock sub-plots sampled during the study (Kolmogorov-Smirnov test for paired samples;  $D_{\max} = 0.138$ ;  $P = 0.576$ ). This result indicates that *T. miliaris* tadpoles use the rock slopes in proportion to their occurrence in their habitat.

Our data on tadpole orientation in relation to direction of water flow indicated that the mean angle was 3° and the length of the mean vector  $r = 0.61$  (Fig. 2). The Rayleigh test ( $z = 13.536$ ;  $P < 0.001$ ) showed that tadpoles of *T. miliaris* do not orient their bodies at random but tend to orient into the water flow in the small drainages (Fig. 2). There is limited information on orientation by larval amphibians (e.g., Goodyear and Altig, 1971; Justis and Taylor, 1976), but most observations concern orientation in relation to light (phototaxis) and temperature. These are behavioral adaptations that enable larvae to develop successfully in various aquatic habitats (Duellman and Trueb, 1994). In the case of tadpoles of *T. miliaris*, orientation is apparently strongly related to the direction of water flow. This observation was reported previously by Bokermann (1965), although without quantification. It is not currently known whether this orientation reduces body resistance against water flow

(Bokermann, 1965) or whether it is a mechanism to improve oxygen consumption.

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### Varanoid-Like Dentition in Primitive Snakes (Madtsoiidae)

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Several recent studies (Estes et al., 1988; Schwenk, 1988; Cooper, 1997; Lee, 1998; Lee and Caldwell, 2000) have suggested that snakes are related to anguimorph lizards and, in particular, are nested within varanoids. However, there are characters that contradict this arrangement. For instance, the teeth of all varanoid lizards exhibit a distinctive infolding of dentine and enamel near the base of the tooth crown. This results in a characteristic “fluted” appearance, with regular vertical grooves and ridges around the entire circumference of the tooth base (Bullet, 1942). The occurrence of this “plicidentine” has previously been interpreted as a derived character uniting varanoid lizards as a monophyletic group, to the exclusion of other squamates such as snakes (Pregill et al., 1986; Estes et al., 1988; Lee, 1998). It characterizes all extant varanoid lizards (*Varanus*, *Lanthanotus*, and *Heloderma*) and extinct terrestrial forms such as *Estesia*.

Until recently, plicidentine has not been observed in nonvaranoid lizards, apart from a weak development in some extinct anguids and necrosaurids, which are closely related to varanoids (Estes et al., 1988). It is also absent in amphisbaenians and has been regarded as absent in all primitive living snakes (e.g., Estes et al., 1988). However, an enigmatic Eocene snake (*Archaeophis*) and some advanced snakes (caenophidians) possess externally fluted or grooved teeth, which are superficially similar to those of varanoids (Janensch, 1906; Bogert, 1943; Klauber, 1956; Vaeth et al., 1985). Nevertheless, despite these occurrences, the apparent absence of plicidentine in more basal snakes (scoleophidians and “anilioids”) has led to the widespread assumption that it is primitively absent in snakes. Here, we describe jaw fragments of two species of primitive snakes (from the extinct family Madtsoiidae) that clearly exhibit varanoid-like plicidentine. These observations, together with other recent studies of primitive snakes (see later), suggest that plicidentine

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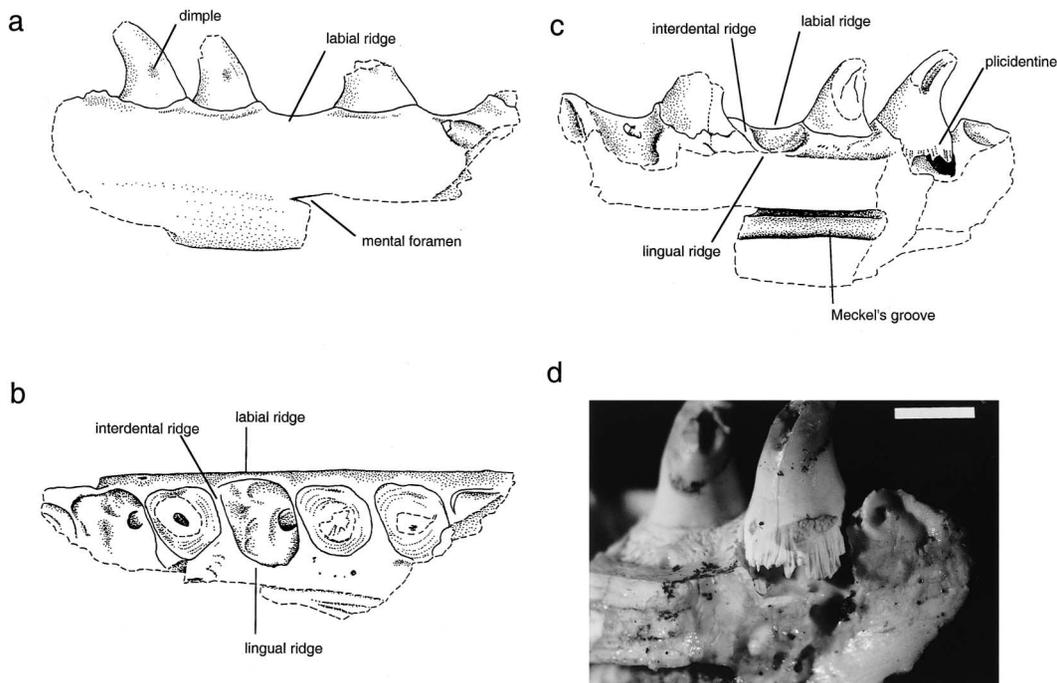


FIG. 1. Right dentary fragment of *Yurlunggur* sp., QMF 36441. (a) Lateral view; (b) dorsal view; (c) medial view; (d) posteromedial view showing zone of infolding (plicidentine) around base of tooth undergoing resorption. Fragments adhering to surface of tooth at upper edge of folded zone indicate extent of bone of attachment. Scale = 5 mm.

is widespread in basal snakes and, therefore, cannot be used to exclude snakes from Varanoidea. It also supports the view that madtsoiids are very primitive snakes rather than close relatives of pythons (McDowell, 1987; Scanlon and Lee, 2000; contra Underwood, 1976; Rage, 1984).

The first specimen (Queensland Museum Palaeontology, QMF 36441) is from RV Site, Godthelp Hill, Riversleigh, northwestern Queensland, Australia ("Tertiary System B," Late Oligocene or Early Miocene; Archer et al., 1997). It is part of the right dentary of a large snake (Fig. 1). No other snake material has yet been identified from RV Site, but vertebrae of the madtsoiid *Yurlunggur* are known from contemporaneous nearby deposits at Riversleigh (e.g., CS, MM, and DT Sites; Scanlon, 1996). The relatively wide and shallow cross-section of the bone (Fig. 1a–c) resembles dentary material of the madtsoiids *Yurlunggur* and *Wonambi*. It is distinct from jaws of pythons (the only other large snakes present in the Australian Tertiary), which are deeper in proportion to width. Furthermore, the size of the dentary matches the vertebrae of *Yurlunggur*. The preserved fragment, with five complete and two incomplete alveoli, is 31.5 mm in length. Complete dentaries of Australian madtsoiids have 17 to 25 alveoli (Barrie, 1990; Scanlon, 1997). Thus, the total length of the dentary was probably around 80 to 130 mm. Dentaries of madtsoiids represent slightly more than half the total mandible length (e.g., Barrie, 1990; Scanlon, 1997). The mandible (and thus head) length of this snake can therefore be estimated as be-

tween 150 and 250 mm. This is consistent with the size of the vertebral elements of *Yurlunggur*, which indicate a snake approximately 6 m long; other Riversleigh madtsoiid taxa attain less than half these dimensions. *Wonambi barriei* is present in some of these sites (Scanlon, 1996; Scanlon and Lee, 2000), but based on its vertebral dimensions, and associated cranial and postcranial elements of *W. naracoortensis* in Pleistocene deposits (Barrie, 1990; Scanlon and Lee, 2000), mandible length in this species would not have exceeded 80 mm. On these criteria, the specimen is assigned to *Yurlunggur* sp. indet.

This fragment comprises approximately the middle third of the dentary, bearing five complete alveoli and portions of two others, one at each end. Teeth remain ankylosed to the second, fourth, and fifth complete alveoli (counting anteroposteriorly), with the relative stages of ankylosis and resorption conforming to the "alternate" replacement mode typical of varanoids and snakes (Edmund, 1969). All tooth crowns are broken distally, the posteriormost being the most complete. Part of a lateral (mental) foramen is preserved below the third complete alveolus, continuous with the mandibular canal, which is exposed medially by breakage. Tooth bases and empty alveoli are roughly trapezoidal in horizontal section, with a relatively sharp (acute) angle anterolaterally (proximolabially) and maximum diameter of approximately 6.5 mm. There is a shallow dimple-like concavity on the labial (lateral) surface of each tooth (Fig. 1a), which has not been observed in other material. The teeth are anky-

losed by bone of attachment to the inner margins of shallow alveoli, as in most snakes. In other squamates, alveoli are either absent (amphisbaenians, most lizards) or are very deep and occupied by large bony pedestals underneath the tooth crowns (mosasauroids). Zaher and Rieppel (1999) have recently questioned the presence of discrete alveoli in mosasauroids, stating that their teeth are "in basal contact with one another" (p.6, repeated on p.12) and "not located in discrete sockets" (p.12). However, mosasauroid teeth are typically not in basal contact with each other but are separated by ridges. These ridges are very high, histologically continuous with the rest of the tooth-bearing element, and persist even after the teeth are shed (e.g., see their fig. 3, and photographs in Lingham-Soliar and Nolf, 1989; Lingham-Soliar, 1994), indicating they are true interdental ridges dividing the dental groove into discrete alveoli, rather than residual bone of attachment (M. W. Caldwell, L. A. Budney, and D. O. Lamoureux, 2000, unpubl.).

Interpretation of the element as representing the middle of the dentary is based on comparison with the complete dentary of *Yurlunggur* (Scanlon, 1996, 1997). The tooth row and lateral margin of the fragment are nearly straight in dorsal view, whereas noticeable curvature would be expected near the posterior (distal) and anterior (mesial) ends of the element. The dentary is deeper than the length of the teeth in the region of the preserved fragment, which again precludes an anterior position. There is also no obvious size gradient, nor any sign of the posterior lateral fossa of the dentary. Alveolus size increases anteriorly and decreases posteriorly in *Yurlunggur* (Scanlon, 1997) but remains relatively constant in the middle of the dentary. The presence of the mental foramen does not imply a more anterior position, as up to three well-spaced mental foramina occur in madtsoiid dentaries (Hoffstetter, 1960; Scanlon, 1997; Rage, 1998).

As in most snakes, the preserved teeth taper sharply from the base and are strongly recurved posteromedially. No indications of carinae are visible on the anterolateral or posteromedial surfaces of the preserved sections of the teeth. However, comparison with complete teeth of madtsoiids (Barrie 1990; Scanlon, 1996, 1997) and other snakes suggests that these ridges were present on the missing distal parts.

The posterior break through the alveolar margin of the dentary exposes the base of the last tooth. This tooth was undergoing resorption of ankylosis, as indicated by a narrow fissure on the occlusal surface between the tooth and its surrounding bone of attachment, a large lacuna within the alveolus extending above and below the tooth base, and the tapering and irregular margin of the base of the tooth projecting freely into this lacuna. The basal part of the tooth clearly shows parallel vertical ridges and grooves formed by infolding of the dentine and enamel (Fig. 1c-d).

A more anterior break, through the tooth in the second complete alveolus, shows the same fluted structure in internal view (schematic view, Fig. 2). Thus, as in "true" plicidentine, the infolding affects both the external and internal surfaces. This tooth is at an earlier stage in its attachment cycle. There is a much smaller lacuna within the alveolus, and the tooth base

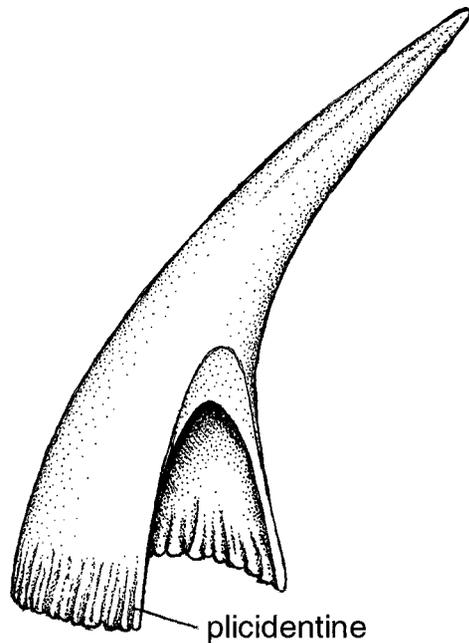


FIG. 2. A reconstruction of a madtsoiid tooth with plicidentine, based on specimens shown in Figures 1 and 3, and additional material discussed in the text.

is much thicker and in extensive contact with the bone of attachment.

This snake thus possessed well developed plicidentine, but it is largely restricted to the part of the tooth normally hidden within the alveolus and completely covered by bone of attachment. The observation of plicidentine in ankylosed teeth, in this case, has depended largely on fortuitous breaks exposing the basal portion of the teeth.

A second madtsoiid specimen is known in which the basal part of an ankylosed tooth is exposed by breakage, and also displays plicidentine. This is the posterior part of a left maxilla of the Pleistocene species *Wonambi naracoortensis* (Flinders University, FU 1762) and comes from the type locality, Victoria Fossil Cave, Naracoorte, South Australia. *Wonambi naracoortensis* is the only large snake known from the deposit (elapids of the extant genera *Pseudechis*, *Pseudonaja*, and *Notechis* are also abundantly present, but much smaller: Smith, 1976; JDS, pers. obs.). Comparison with complete maxillae of *W. naracoortensis* from Henschke's Cave, Naracoorte (Barrie, 1990; Scanlon and Lee, 2000) reveals an almost perfect match, and further supports referral to this taxon.

The specimen is 50 mm long and comprises the posterior end of the left maxilla (Fig. 3a). The anterior three alveoli are only partly preserved because of an oblique fracture, the remaining 10 alveoli are complete. Complete maxillae of two *Wonambi* individuals from Henschke's Cave, Naracoorte (Barrie, 1990; Scanlon, 1996) are 70 and 81 mm long, with 23 and 22 alveoli, respectively. Comparison suggests a total maxilla length of about 97 mm for the present specimen, and mandible length of 160 mm (cf. figures in Barrie, 1990;

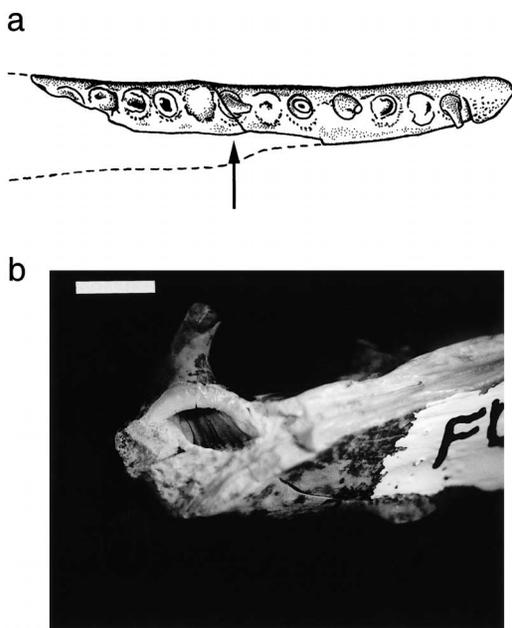


FIG. 3. Left maxilla fragment of *Wonambi naracoortensis*, FU 1762. (a) Ventral (occlusal) view of whole specimen (scale = 10 mm), with arrow indicating oblique break. (b) Medial view of broken surface indicated by line in (a), showing zone of infolding (plicidentine) on internal surface of broken tooth (scale = 3 mm).

Scanlon and Lee, 2000). This size difference is consistent with the observation that the largest known vertebrae from Victoria Cave are also somewhat larger than those from Henschke's Cave (Smith, 1976; Barrie, 1990; JDS, pers. obs.). The medial edge is damaged except for the region adjacent to the four most posterior alveoli, and posterior to the last alveolus where the surface is sculptured, apparently for tendinous muscle insertion (m. pterygoideus). Another fresh transverse break passes obliquely through the base of a firmly ankylosed tooth (seventh from anterior end). The medial internal view of the tooth base (Fig. 3b) shows infolding similar to the *Yurlunggur* specimen.

Resorption of part of the tooth base before replacement is typical of most squamates, including snakes (Edmund, 1969). That plicidentine has been observed only in ankylosed teeth of madtsoiids, and not in any of the numerous isolated (shed) teeth found at Riversleigh and Naracoorte, suggests that the basal (plicidentine) portion of the tooth is usually completely resorbed before shedding. If such a pattern were widespread among snakes, including recent forms, the occurrence of a basal zone of plicidentine (mostly hidden within the alveolus and resorbed before teeth are shed) could easily have escaped notice. The possibility that plicidentine is present but unrecorded in many extant snakes is suggested by the observation that plicidentine-like infolding is clearly visible on the fangs of some elapids, which are unusual in that their alveoli are not completed by bone posteriorly (Bogert,

1943; pers. obs.). This infolded basal part, visible when ankylosed, is resorbed before the fang is shed.

The distribution of plicidentine in basal snakes is of interest. It was originally proposed that pachyophiids, that is, *Pachyrhachis* and *Pachyophis*, must lie outside of all other snakes (Haas, 1979, 1980a,b). Haas noted that pachyophiids displayed a curious mosaic of snake-like and varanoid-like features: although he was unsure whether they were truly related to snakes, or were convergent, he was certain that the primitive (varanoid-like) features indicated they were not nested within extant snakes. Zaher and Rieppel (1999) and Tchernov et al. (2000) have recently challenged Haas' conclusion and instead suggested that pachyophiids are advanced (macrostomatan) snakes. However, a more comprehensive recent study of snake relationships confirms that pachyophiids are basal to all other snakes; other basal lineages of snakes identified include madtsoiids, *Dinilyisia* and scolecophidians (Scanlon and Lee, 2000; for review, see Coates and Ruta, 2000). Pachyophiids possess weakly infolded teeth, at least on the external surfaces (Lee and Caldwell, 1998); these were identified in the original descriptions but interpreted as possible venom grooves (Haas, 1979, 1980a). The present study reveals that madtsoiids possessed plicidentine. Only a few teeth are preserved in *Dinilyisia*, at the posterior end of the maxilla (Estes et al., 1970), and the condition in this taxon cannot be ascertained. Scolecophidians, however, lack plicidentine. Both species of *Archaeophis*, which also appear to be rather basal snakes (Scanlon, 1996), also have multiple ridges and grooves on the tooth crowns (Janensch, 1906; Tatarinov, 1988) resembling plicidentine. The presence of plicidentine in *Pachyrhachis*, madtsoiids, and *Archaeophis* means that it is probably primitive for snakes. Thus, absence of plicidentine can no longer be used as a character excluding snakes from Varanoidea. If recent studies indicating that snakes are nested within varanoids are correct (e.g., Schwenk, 1988; Cooper, 1997; Lee and Caldwell, 2000), the most parsimonious assumption is that plicidentine evolved once at the base of Varanoidea and that its presence in basal snakes represents direct retention of the varanoid condition (Fig. 4). However, plicidentine is absent in almost all modern snakes (i.e., scolecophidians, anilioids, booids, and caenophidians) surveyed for this study (see Appendix 1); the exception was the caenophidian *Cerberus*. A few occurrences have been noted in various other caenophidian lineages (Bogert, 1943; Klauber, 1956; Vaeth et al., 1985). Although a more thorough survey is required, the most parsimonious interpretation is that plicidentine was lost at the base of modern snakes, and its presence within caenophidians is a reacquisition.

A possible reason for loss of plicidentine at the base of modern snakes involves allometry and paedomorphosis. Varanoid lizards, *Pachyrhachis*, madtsoiids, and *Archaeophis* are moderately to extremely large, and possess plicidentine. However, basal lineages of modern snakes (scolecophidians and anilioids) are all much smaller. The amount of infolding is highly size-dependent in extinct "labyrinthodont" amphibians (e.g., Bystrow, 1938; Warren and Davey, 1992; A. Milner, pers. comm.) and living varanoids (MSYL, pers. obs.). Teeth which are larger have more pronounced infoldings, and this trend occurs both within and be-

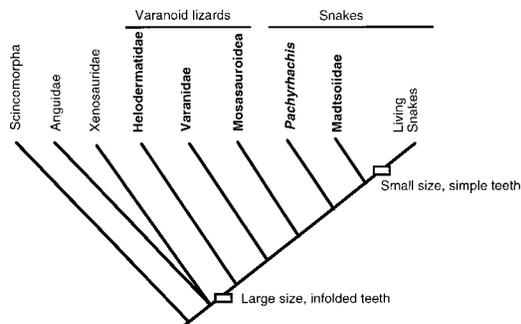


FIG. 4. The distribution of plicidentine in varanoids and primitive snakes, and its phylogenetic implications. The taxa with plicidentine are shown in bold, taxa without plicidentine are shown in normal font. As discussed in the text, the condition in the primitive snake *Dinilyisia* is not known, whereas the position of the fossil snake *Archaeophis* is not yet clear: neither is shown on the diagram. The "lizard" portion of this tree is based on Schwenk (1988) and Lee (1998); the snake portion is based on a recent phylogenetic analysis of 234 morphological characters in all the major fossil and living lineages of snakes (Scanlon and Lee, 2000). The position of pachyophiids outside of all other snakes is provisionally accepted (Haas 1979, 1980a,b; Scanlon and Lee 2000; but see Zaher and Rieppel 1999).

tween species. The basal zone with plicidentine is the last part of the tooth crown to be formed and would be expected to be less apparent in small teeth which cease developing early. Thus, the loss of plicidentine in snakes above *Pachyrhachis*, madtsoiids, and presumably *Dinilyisia* might have been associated with size reduction. The ability to develop this trait might have been lost by the time certain forms (boines, pythones) again reevolved large size. Evolution by heterochrony could also account for zones of infolding or carinae appearing on the upper part of the crown in various snakes and mosasauroids, as well as (or instead of) on the tooth base; the fluted or striated tooth crowns of some caenophidians might have arisen in this way (Vaeth et al., 1985), but a wide survey of tooth base morphology (not closely examined by Vaeth et al., 1985) would be required to test this idea.

Alternatively, it might be suggested that plicidentine evolved repeatedly in squamates and was never present in the ancestor of snakes. This hypothesis is unparsimonious if snakes are accepted as nested within varanoid lizards (Fig. 4), because it would imply at least three origins within varanoids and two within snakes. Also, this convergence would be difficult to explain biomechanically. The varanoid origins would have been associated with strongly oblique, pleurodont attachment of teeth, whereas in snakes the angle, depth of alveoli, and extent of bone of attachment are all quite different (though similar in some respects to conditions in mosasaurs; Edmund, 1969). The functional significance of plicidentine does not appear to have been rigorously analyzed biomechanically. One possibility is that the basal infoldings provide a greater area of bone surface and thus strengthen the tooth

base and the tooth-to-jaw attachment. Another possibility is that the infoldings allow slight flexibility in the tooth base (analogous to movements in a concertina); this bending would help absorb shock during bites. In all these models, the forces encountered by the tooth base would presumably scale according to tooth (and skull) volume but the amount of bone in the tooth base would only scale according to tooth surface area (assuming enamel thickness remains constant), thus requiring greater infolding in larger teeth. These models are all consistent with the prevalence of plicidentine in large, kinetic ("snap") feeding predators such as temnospondyls, varanoids and snakes, where the teeth would presumably experience large shock forces during jaw closure.

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#### APPENDIX 1

##### Material Examined.—Institutional Abbreviations:

AMS—Australian Museum, Sydney; QM—Queensland Museum, Brisbane.

Scolecophidia: *Ramphotyphlops* sp. QM J5326; Anilioidea: *Cylindrophis maculatus* AMS R13156; Booidea: *Aspidites melanocephalus* QM J5616, QM J30786, *Morelia viridis* QM J15815, *Liasis fuscus* QM J226898, *Liasis olivaceus* QM J28903, *Morelia amethystina* QM J15815, *Morelia spilota* QM J62828; Caenophidia: *Acrochordus arafurae* QM J23718, *Boiga irregularis* QM J 22173, *Cerberus*

*australis* QM J23630, *Dendrelaphis punctulatus* QM J47449, *Notechis scutatus* QM J28404, *Oxyuranus scutellatus* QM J45304, *Pseudechis colletti* QM J6280, *Pseudechis australis* QM J29006, *Pseudonaja textilis* QM J26542, J 26668, *Tropidonophis mairii* QM J24550.

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### Fecal Testosterone Concentrations May Not Be Useful for Monitoring Reproductive Status in Male Blue-Tongued Lizards (*Tiliqua nigrolutea*: Scincidae)

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Assessment of reproductive status in animals generally depends on monitoring hormone concentrations in plasma, but blood sampling often involves significant stress to the subject. Monitoring steroid profiles by assaying excreted steroids in urine or fecal samples is noninvasive but does pose some problems. Unlike plasma assays, urinary and fecal steroid analyses are of relatively little value in monitoring rapid, short-term changes in hormone concentrations (Heistermann et al., 1993) because there is a significant delay between production and excretion of steroids. However, such assays do enable measurement of “pooled” hormone concentrations over time (Wildt et al., 1995; Brown, 1997).

Hormones do not usually appear unchanged in the urine or feces but are present as a range of metabolites produced by the liver, the major site of steroid metabolism (Kime, 1987). The proportions and forms of steroid excreted differ between the two excretory pathways and may be highly variable between species (Aldercreutz et al., 1980). In the maned wolf (*Chrysocyon brachyurus*), 97% of testosterone (T) is excreted as unconjugated metabolites in the feces (Velloso et al., 1998), whereas in African wild dogs (*Lycan pictus*), 60% of steroids are excreted in the feces and 40% in the urine (Monfort et al., 1993). A proportion of the excreted steroid is present as water-soluble conjugates—glucuronidates and sulfates—which can complicate hormone analysis. In most mammals, however, voided feces contain a higher percentage of free than conjugated steroids (Adams et al., 1994; Wasser et al., 1996; Velloso et al., 1998), so excreted steroids are measurable by standard assay procedures. However, even if the same hormones are produced, different liver enzymes may produce different metabolites for excretion (Hodges, 1986); fecal steroid assays, therefore, need to be validated separately for each species.

Most studies of fecal steroids have been conducted in mammalian species (Schwarzenberger et al., 1996),

with limited work on birds such as the kakapo (*Strigops habroptilus*; Cockrem and Rounce, 1995). This critically endangered, cryptic species is not amenable to invasive monitoring strategies, but Cockrem and Rounce (1995) have successfully used the ratio of fecal T to estrogen to assign sex in kakapo. Fecal steroid monitoring has also been used to follow the reproductive cycle in Japanese crested ibis (*Nipponia nippon*) and rock ptarmigan (*Lagopus mutus*; Kikuchi and Ishi, 1997) and in the northern spotted owl (*Strix occidentalis caurina*; Wasser et al., 1997; Brown et al., 1995).

There are, however, only two published reports of the application of fecal steroid monitoring to reptiles. The reproductive cycles, characterized by behavioral observations and ultrasound imaging, of four species of tortoise (*Geochelone elephantopus*, *Geochelone gigantea*, *Testudo graeca*, *Testudo hermanni*) have been positively correlated with profiles of fecal steroid metabolites (Casares, 1995; Dobeli et al., 1992). Ideally, fecal steroid profiles should be validated against plasma steroid profiles for the same species (Pryce et al., 1993), but this was not achieved for the tortoises. No squamate reptile species has yet been monitored using fecal steroid assays, and further study of the potential of such techniques for monitoring reptilian reproductive cycles is clearly required.

Working from the premise that new techniques are best developed using a relatively common species, we collected fecal samples opportunistically during a study of the annual reproductive cycle and plasma concentrations of steroid hormones in male blotched blue-tongued lizards (*Tiliqua nigrolutea*; Edwards and Jones, 2001). Here we report on the development of a method for assay of T in feces of this species. We compare the profile of fecal T with that for plasma T through the reproductive cycle of blotched blue-tongued lizards and also examine the relative proportions of conjugated and free T in feces.

We collected fecal samples from captive male *T. nigrolutea* from March 1997–February 1999. The wild-caught animals were held at the University of Tasmania in semi-natural conditions. Male and female lizards cycle in synchrony with the wild population (Edwards, 1999; Edwards and Jones, 2001); details of animal husbandry are given in Edwards and Jones (2001). We collected fecal samples ( $N = 29$ ) passed voluntarily while animals were being observed for reproductive behaviors. These samples were classified according to reproductive stage, and mean fecal T levels (Fig. 1) were calculated using results obtained by assaying these samples. Plasma T values were derived independently from the more comprehensive dataset presented as monthly mean values in Edwards and Jones (2001); standard errors are not, therefore, presented (Fig. 1).

Upon collection, each fecal pellet was frozen at  $-20^{\circ}\text{C}$  in an individual, snap-lock bag until analysis. Prior to extraction of steroids, we thawed the fecal samples and mixed each one well to control for possible heterogeneous steroid distribution within the pellet. Samples were then lyophilized for 24 h to control for varying water content (Wasser et al., 1994; Thompson et al., 1998). The lyophilized samples were ground and the fecal material separated from dried plant material and snail shells to produce a powder ready for extraction. We used the fecal steroid extrac-

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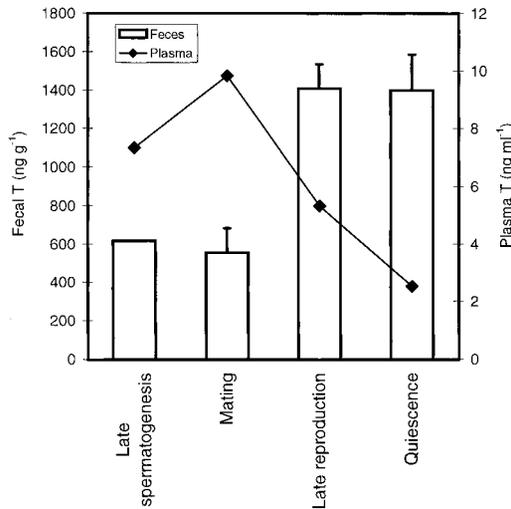


FIG. 1. Comparison of mean fecal testosterone (T) concentrations ( $\text{ng g}^{-1} \pm \text{SE}$ ) and plasma T concentrations ( $\text{ng ml}^{-1}$ ) in male blue-tongued lizards (*Tiliqua nigrolutea*) at four stages of the annual reproductive cycle. (Late spermatogenesis: September,  $N = 1$ ; Mating period: October–November,  $N = 9$ ; Late reproductive period: December,  $N = 3$ ; Reproductive quiescence: January–March,  $N = 8$ .) Mean plasma T concentrations are derived from data presented in full in Edwards and Jones (2001); for clarity, error bars are not shown.

tion method of Wasser et al. (1994), but some critical modifications were required. Initially our samples contained unknown, hydrophobic, interfering substances that resulted in very high nonspecific binding values ("blanks") for samples in the radioimmunoassay (RIA). We needed to include purification steps additional to those described by Wasser et al. (1994) to reduce the assay blanks to acceptable levels (i.e., equivalent to the RIA nonspecific binding).

For each sample, 0.1 g of fecal powder was boiled in 5 ml of 90% ethanol (Brown et al., 1994); 50  $\mu\text{l}$  of tracer [ $^3\text{H}$ ]T: 4400 cpm] was added to the sample prior to extraction to measure recovery. The sample was boiled for 20 min then centrifuged at 1500 g for 15 min. The supernatant was collected into a clean tube, and the pellet washed with 3 ml of 90% ethanol, vortexed well, and centrifuged at 1500 g for 5 min. Washes were dried down, and then the sample was reconstituted by standing for 24 h in 1 ml methanol, with frequent vortexing, at 4°C.

At the second stage of the extraction, 0.5 ml of reconstituted sample was moved to a clean tube, dried down and reconstituted in 1 ml of 2% ethanol in hexane for 24 h. After centrifuging for 5 min, the supernatant was collected; the "pellet" was rinsed with 1 ml of 2% ethanol in hexane and then centrifuged and the supernatants combined, dried down, and reconstituted in 1 ml of 2% ethanol in hexane for 24 h. For the final purification step, we used Sep-Pak C18 cartridges (Waters) prewetted with 5 ml 2% ethanol in hexane. The sample was applied to a column and eluted with 1 ml of 2% ethanol in hexane. The eluate was

dried down and reconstituted for 24 h in 1 ml 2% ethanol in hexane.

Duplicate subsamples (50  $\mu\text{l}$ ) of the final extract were assayed for T by standard RIA, using ( $^3\text{H}$ )T as label, and charcoal separation, as detailed in Swain and Jones (1994). The cross-reactivity of the antiserum with T, dihydrotestosterone, and androstenedione is 100%, 28.6%, and 6.2%, respectively, and < 2.5% with all other steroids tested, including epitestosterone (Bradley, 1990). This assay has been validated for assay of T in plasma of *T. nigrolutea* (Edwards and Jones, 2001). We included duplicate assay blanks (i.e., tubes without antiserum) for every sample to measure sample nonspecific binding; these counts were subtracted from the sample counts before calculation of assay results. Serial dilutions of fecal extract ran parallel to the standard curve suggesting that T was measured accurately. The sensitivity of the assay is 15 pg T; the intraassay coefficient of variation for the fecal steroid assay is 5%, whereas the interassay coefficient of variation is 13%.

To determine the proportions of conjugated and free steroids in the feces, we used the ether/water extraction method of Velloso et al. (1998). Four fecal samples containing high concentrations of T were lyophilized and extracted as above. After the column purification step, the extracted steroids were redissolved in 1 ml distilled water combined with 10 ml ether: this separated conjugated, water-soluble steroid from unconjugated (free) steroid. The tubes were placed in a  $-20^\circ\text{C}$  freezer for 15 min to freeze the lower aqueous layer; then the upper ether fraction was decanted into clean tubes. We dried both fractions completely and resuspended them in 1 ml methanol before taking subsamples for RIA of testosterone. Data are reported as means  $\pm$  SE.

We were able to detect T in all fecal samples analyzed. The overall mean recovery (extraction efficiency) was 25% ( $N = 32$ ). Fecal T concentrations ranged from  $\sim 500 \text{ ng g}^{-1}$  to  $\sim 1500 \text{ ng g}^{-1}$  of dry feces. The mean fecal T concentrations for each reproductive stage are shown in Figure 1. The mean fecal T concentration varied significantly between reproductive stages (ANOVA:  $F_{3,17} = 5.58$ ,  $P = 0.0075$ ); however, contrary to expectation, fecal T concentrations were lowest ( $554 \pm 129 \text{ ng g}^{-1}$  of dry feces) in animals during the mating period, rising significantly to  $1398 \pm 187 \text{ ng g}^{-1}$  in reproductively quiescent animals. Differential ether/water extraction revealed that  $12 \pm 3.3\%$  of the fecal T measured by RIA was free T associated with the organic solvent, with  $88 \pm 13\%$  of T associated with the aqueous phase, and presumably representing conjugated steroid.

Thus, our results show that it is possible to measure T in feces of male blue-tongued lizards. Testosterone was detected in all samples assayed, and fecal T concentrations ranged from  $\sim 500 \text{ ng g}^{-1}$  to  $\sim 1500 \text{ ng g}^{-1}$  dry feces. These concentrations are high compared with those reported for some other species, suggesting that the majority of T is excreted via this route; similarly, the gut is the major route of steroid excretion in *Tiliqua rugosa* (Bourne, 1972). By comparison, fecal T concentrations range from  $< 60 \text{ ng g}^{-1}$  to a peak of  $441 \text{ ng g}^{-1}$  in the kakapo (Cockrem and Rounce, 1995) and from  $400\text{--}800 \text{ ng g}^{-1}$  in the male maned wolf (Velloso et al., 1998). In male siberian polecats

(*Mustela eversmanni*) concentrations of T in the feces are similar to those in *T. nigrolutea*, ranging from 200 ng g<sup>-1</sup> to about 1000 ng g<sup>-1</sup> (Brown, 1997). Fecal T concentrations in four species of tortoise ranged from 25 ng g<sup>-1</sup> to 694 ng g<sup>-1</sup> (Dobeli et al., 1992; Casares, 1995) with high intraindividual variation. Plasma hormone cycles were not reported, but the patterns of variation in fecal T concentrations appeared to show some correlation with reproductive activity. In contrast, our results suggest that there is an inverse relationship between concentrations of T in feces and in plasma of male blue-tongued skinks, with fecal T concentrations being highest during the nonbreeding season, when plasma T levels are low (Fig. 1). The only published report of a similar occurrence is in the African elephant, in which fecal estrogens increase late in gestation (Fieß et al., 1999) while plasma concentrations are decreasing (Hodges et al., 1983).

There are a number of factors that may contribute to this apparently anomalous result. Correlation of fecal and plasma hormone concentrations may be inherently more problematic in ectotherms (particularly temperate species), than in birds and mammals. In reptiles, gut passage time varies through the year (McKeown, 1996), and therefore rates of excretion may also vary. For another study, we fed female blue-tongued lizards cat food colored with food dye. During late summer, they passed no more than two fecal pellets per week with some dye remaining in the pellets for at least a week, but during spring, pellets were passed daily (NA and AE, pers. obs.). Male blue-tongued skinks reduce their feeding rates markedly during autumn and cease feeding several weeks before hibernation. A slower gut passage time, resulting in accumulation of excreted steroid, could therefore explain the higher concentrations of T in feces during this quiescent period.

Also, temperature influences steroid metabolism profoundly through thermal effects on enzyme activity (Kime and Hyder, 1983; Bourne et al., 1986; Huf et al., 1989). Particularly in temperate reptiles, variation in mean body temperature through the annual cycle may be reflected in changing patterns of steroid excretion through thermal effects on steroid metabolism. For example, a change in the rate of catabolism or clearance of hormone from the plasma would also affect its rate of excretion. Finally, fecal steroid concentrations do not show a distinct annual pattern in all species. For example, in maned wolves there is no clear annual cycle of fecal T, although fecal T concentrations have been used to distinguish between the sexes (Velloso et al., 1998).

Over 80% of fecal T was present as immunoreactive conjugates in *T. nigrolutea*, suggesting that limited deconjugation occurs in the gut in this species. In blue-tongued lizards, testicular tissue from quiescent males produces a larger proportion of conjugated steroids than tissue from reproductively active males (Edwards, 1999). Seasonal variation in rates of testicular steroid conjugation has been reported for some other reptilian species (Huf et al., 1987). Further studies may reveal variations in the proportion of conjugated T in feces at different times of year.

Our results suggest, therefore, that assay of fecal T concentrations may not be useful for assessing reproductive status in blue-tongued lizards because of the

lack of correlation between T concentrations in feces and plasma. As our experience has illustrated, extraction of fecal steroids is problematic in some species. Exploratory studies of fecal steroids in varanids at San Diego Zoo have proved unsuccessful (V. Lance, pers. comm.). For both Japanese crested ibis and rock ptarmigan, extraction of feces using standard organic extraction methods resulted in extracts containing a hydrophobic substance that interfered with RIA of fecal steroids (Kikuchi and Ishii, 1997); for each species, different chromatographic techniques were needed to remove the interfering substance. Our samples similarly contained unidentified interfering substances that were eliminated only by a complex series of purification steps that reduced the extraction efficiency. Further work is needed to investigate the usefulness of fecal steroid monitoring in reptiles, and to identify the physiological reasons for the pattern of fecal T concentrations observed in this study.

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