Discussion

The new records reported here together with the recent works by Day (1990) and Lim and Lim (1999) indicate that the herpetofauna of Pulau Tioman almost certainly remains incompletely known. The steep, mountainous interior and rocky coastline of this island are relatively inaccessible and large tracts of forest remain unexplored. Thus, it is probable that a number of forest-dwelling species, especially small secretive taxa, remain to be discovered. Equally important is the fact that many of the reported species are known from only one or two specimens, and some of these, as well as others known from larger series, show morphological differences from their mainland counterparts (Day 1990; Hendrickson 1966a,b; Lim and Lim 1999). Thus, it is important that series of specimens of each species be obtained and that appropriate morphological and molecular comparisons be made to their Malayan and Bornean counterparts to assess the actual levels of diversity and endemism. As previously noted, potentially undescribed species include *Rana* sp. (Hendrickson 1966b), *Cnemaspis* sp. (Das and Grismer, *in prep.*), *Dibamus* cf. *alfredi*, *Oligodon signatus* (Day 1990), and *Trimeresurus popeiorum* (see Lim and Lim 1999). Known endemics include *Ansonia tiomanica* (Hendrickson 1966b) and *Cyrtodactylus tiomanensis* (Das and Jim 2000).

Acknowledgments.—For comments on the manuscript and identification we thank I. Das. This work was supported by the Presidential Grant from La Sierra University awarded to LLG.

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TECHNIQUES

Herpetological Review, 2002, 33(1), 29–32.

In situ Measurement of Larval Salamander Growth Using Individuals Marked with Acrylic Polymers

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Mark-recapture studies are often used to provide valuable life history information for animal populations. However, long-term marking of larval amphibians has been problematic because of their small size, delicate skin, and ability to regenerate tissues (Cecil and Just 1978; Donnelly et al. 1994; Seale and Boraas 1974). Procedures that have been used to mark larvae include fin-clipping (Turner 1960), whole-body staining with neutral red (Guttman and Creasey 1973; Herreid and Kinney 1966), injection of mineral oil and petroleum jelly mixtures (Seale and Boraas 1974), application of fluorescent pigments with gas pressure (Ireland 1989; Taylor and Deegan 1982) or heat brands (Ireland 1973), and application of a Congo red and dimethyl sulfoxide paste (Ireland 1989). However, these methods are cumbersome, inhibit growth (Travis 1981), do not produce unique marks, or are only useful for short-term studies. Passive integrated transponder (PIT) tags have been used to mark newts and spadefood toads (Fasola et al. 1993; Jehle and Hodl 1998) as well as metamorphic ambystomid salamanders (Ott and Scott 1999). However, these tags are too large for many larval amphibians. Toe-clipping has been the traditional method for marking amphibians (Ferner 1979), but some larvae may regenerate toes too quickly (<1 mo.) for use in long-term studies. In addition, larvae sometimes suffer toe injuries that make identification difficult (Ott and Scott 1999). As a result of these problems, field experiments that require the individual identification of amphibian larvae have been limited.

Subcutaneous injection of acrylic polymers is a method that has been successful for long-term marking of fish (Kelly 1967; Lotrich and Meredith 1974), adult salamanders (Ireland 1989; Wooley 1973), and tadpoles (Anholt et al. 1998; Cecil and Just 1978). These polymers are non-toxic and the marks can remain visible for a year or more. Each individual can also be given a unique mark by using several different colors and multiple mark locations. This method is efficient and cost-effective. However, it has yet to be tested on larval salamanders.

Larval salamanders are an important component of first order high-gradient streams because they are often the top predators in these fishless systems. The Blue Ridge two-lined salamander, *Eurycea wilderae* Dunn, is the most abundant larval salamander in the headwaters of the study region (Bruce 1985) and is the focus of our study. In these streams, hatchlings of *E. wilderae* appear in April or May, and the larval stage lasts one or two years (Bruce 1982, 1985, 1988; Voss 1993). The specific objectives of our study were to evaluate the polymer injection method in field trials and to use mark-recapture data to determine individual growth rates of larval *E. wilderae*.

This study was conducted in a first order stream that drains catchment 53 at the Coweeta Hydrologic Laboratory, Macon County, North Carolina. Vegetation consists of mixed hardwoods with a dense riparian understory of rhododendron (*Rhododendron maximum*). This stream was treated with the insecticide methoxychlor in 1980 to examine the role of invertebrates in processing organic matter (Cuffney et al. 1984; Wallace et al. 1982; Wallace et al. 1986). However, following treatment, the stream quickly recovered to reference conditions (Lugthart and Wallace 1992; Wallace et al. 1982; Wallace et al. 1997). Detailed descriptions of the Coweeta basin are provided by Swank and Crossley (1988). In addition to *E. wilderae*, four other salamander species are common in the headwaters at Coweeta: *Desmognathus quadramaculatus*, *D. monticola*, *D. ocoee*, and *Gyrinophilus porphyriticus*.

The entire wetted area of a 100-m reach was sampled for *E. wilderae* larvae approximately every month from November 1997 through June 1999. The stream was sampled at night with a head lamp and aquarium dip net (15.5 × 12 cm). Only loose cover objects (e.g., cobble, wood, leaves) were turned over when searching for larvae to minimize disturbance to the stream. Captured larvae were placed in individual 20 mL plastic vials filled with stream water. Vials were then placed on ice in a cooler.

In an on-site laboratory, each larva was anesthetized in Petri dishes containing 0.1% tricaine methylsulfonate (MS-222) (Beachy 1994). Snout-vent length (SVL) was measured to the posterior margin of the vent to the nearest 0.5 mm using a dissecting scope and vernier calipers. The anesthetized larvae were then placed on a damp paper towel under a dissecting scope. A Liquitex® acrylic polymer emulsion (available at most art supply stores) was injected into the tail using a 3 cc syringe with a 26 gauge 16 mm needle as described by Cecil and Just (1978). Initially, six different colors were used: blue, green, red, white, purple, and yellow. However, yellow was abandoned because it was difficult to see. Marks were inserted just under the skin of the tail immediately behind the hind legs. This insertion point left a mark that was visible if the tail was subsequently lost. Two discrete marks could be placed on either side of the tail, a feature that allowed the same color to be used twice on one side (Fig. 1). Marking newly hatched larvae (<14 mm SVL) was more difficult but still possible. After some practice, each larva could be measured and marked within five min. The five colors and four mark locations allowed for ca. 3,000 unique combinations. Following placement of a mark, larvae were revived in stream water, returned to their plastic vials, refrigerated overnight, and released at the point of capture the following morning or evening.

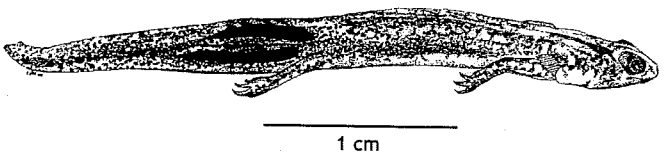

FIG. 1. A marked *Eurycea wilderae* larva demonstrating the location of color bands.

To determine growth, SVL was first converted to ash-free dry mass (AFDM) using a length-weight regression derived for *E. wilderae* in the study stream:

$$M = 0.0023L^{3.09} \quad (r^2 = 0.96, p<0.001, N = 22)$$

where M is larval mass (mg AFDM) and L is SVL (mm) (Lugthart 1991). Ash-free dry mass is a more accurate measure of growth than SVL or wet weight because it accounts for inorganic matter in guts. Individual daily growth rate (g) was then calculated as:

$$g = \ln(M_2/M_1)/t$$

where M_1 = initial larval mass, M_2 = final larval mass, and t = time interval in days (Romanovsky and Polishchuk 1982). Exponential growth was assumed. Average growth was also determined by plotting the mean biomass by sample date beginning after the hatchlings appeared in May 1998. In addition, we tested for differences in mean SVL of marked and unmarked larvae for each sample date throughout the study period using t-tests. We used a Mann-Whitney U-test when data failed to meet the assumption of normality.

To assess mark duration, 20 larvae were collected from an adjacent headwater stream and returned to the laboratory. These larvae were marked following the procedure described above and maintained in an aerated 37.9 L aquarium in a laboratory cold room at 5°C. Though this temperature is lower than the average annual temperature in the stream, these temperatures are encountered in winter and the larvae remain active. Moss-covered stones were collected from the stream and added to the aquarium for refuge. Water was changed weekly and larvae were fed freeze-dried *Daphnia* sp. However, *Daphnia* sp. consumption was not directly witnessed during laboratory study period. Larvae were checked regularly for any fading of marks or mortality. After one year, surviving larvae were returned to the stream where captured.

A total of 428 *E. wilderae* larvae were captured, marked, and released during this study. Ninety-eight individuals were recaptured and used for growth determination (~23% of the total marked). Of the 98 recaptured larvae, 32 were recaptured on more than one sample date. Though growth slowed slightly in the winter, growth was nearly linear over the year (Fig. 2). The average individual daily growth rate for *E. wilderae* larvae was 0.0024 d^{-1} (± 0.0002 S.E).

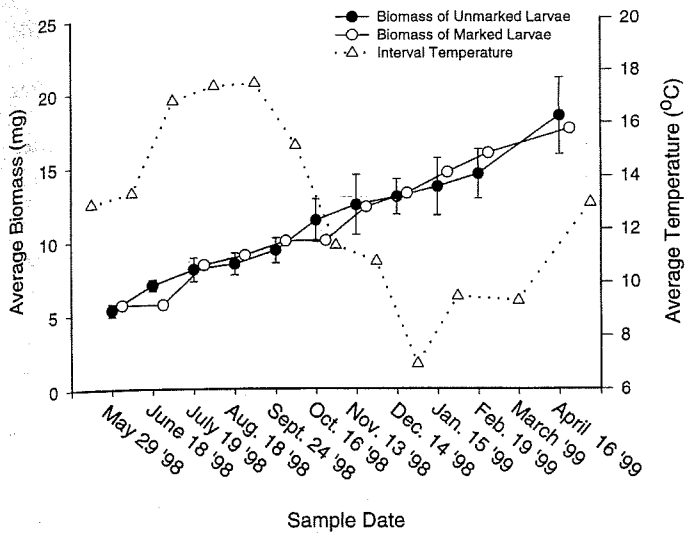

FIG. 2. Average biomass (mg) for all marked and unmarked (± 95% C.I.) *Eurycea wilderae* larvae by collection date beginning when hatchlings first appeared in spring. Stream temperature is plotted as the average between sample events.

Given their delicate appearance, the larvae were surprisingly durable during marking. They typically recovered from the anesthetic within ten minutes of returning to vials of stream water. A poorly placed mark resulted in the death of one larva, but all others were alive at the time of release with no apparent change in behavior from pre-marking. Larvae were recaptured up to eight months after marking (Fig. 3). The average biomass and SVL of marked larvae was not significantly different from unmarked larvae on all but one date ($p>0.05$)(Fig. 2) when recapture sample size was low (N = 1). This indicates that marking had little, if any, effect on growth. In the laboratory aquarium, all 20 marked larvae survived at least six months and 17 survived for a full year. Fading of marks was sometimes evident in both the field and laboratory but individual identification was always possible.

Amphibian larvae typically show reduced growth during winter (Beachy 1997), but our results show that *E. wilderae* growth was nearly linear throughout the year. Beachy (1997) found a similar linear growth pattern for larval *E. wilderae* in cage experiments. Our daily individual growth rate of 0.0024 d^{-1} agrees closely with a previous study that determined *E. wilderae* larval growth using chambers in a nearby headwater stream. In that study, the average individual growth rate from three different field trials was 0.003 d^{-1} (Lugthart 1991). However, results from chamber experiments must be interpreted with caution because larval densities and prey availability can influence growth rate (Petranka and Sih 1986; Scott 1990; Smith 1990).

By using acrylic marks, we were able to determine growth rates for individual, free-ranging larvae. Travis (1981) found that whole body staining with neutral red reduced growth of tadpoles and warned that any marking method might adversely affect growth. However, the acrylic polymers are non-toxic and there were no discernable differences in SVL or biomass between marked and unmarked larvae by sample date. There is therefore no evidence to indicate that marks affected growth.

The high survival rates of marked larvae both in the field (up to eight months after marking) and laboratory (up to one year) also indicated the marks had a negligible effect on survivorship. Our laboratory aquarium survival rate (85% after 1 yr) was much higher than those reported for *Eurycea* spp. in artificial streams over shorter time periods (27% over ~ 4 mos. in Resetarits 1991; 39% over ~2 mos. in Gustafson 1993; and 31% over ~3 mos. in Gustafson 1994). However, our higher survival rate could be partially attributed to reduced metabolic demand associated with lower laboratory temperature. The recapture time interval curve (Fig. 3) is indicative of the Type III survivorship curve (Deevey 1947) that has been previously documented for *E. wilderae* (Bruce 1988). The slope of this line may therefore be attributed to natural mortality or emigration rather than mark effects.

This study demonstrates the effectiveness of subcutaneous acrylic polymer injection for long-term monitoring of larval salamander populations. This method provides a practical and cost-efficient way to conduct field studies of the top predators in small, fishless streams. Because unique marks can be generated quickly, the method can easily be used not only to assess larval growth, but also population size and individual movements. Individual growth data can also be used to determine secondary production (Benke 1984; Waters 1977). Mark-recapture studies offer several additional advantages over cage experiments. For example, growth can be determined under natural conditions with realistic prey levels. In addition, natural movements can be measured and thus add to our understanding of species colonization and dispersal.

According to Ferner (1979) an ideal mark or tag should: 1) not affect individual survivorship or behavior, 2) uniquely identify individuals, 3) remain over the individual's lifetime, 4) be easily discernable, and 5) be easily applied in both field and laboratory studies. In addition, the marking or tagging method should be cost-effective and limit handling time of an individual (Ott and Scott 1999). Acrylic polymer injection meets most of these criteria and is currently the only viable method for *E. wilderae* larvae. A potential problem with this method is that the marks may increase larval visibility and thereby increase predation pressure from larger salamanders such as *D. quadramaculatus, D. monticola,* and *G. porphyriticus.*

Choice of an appropriate marking or tagging method should

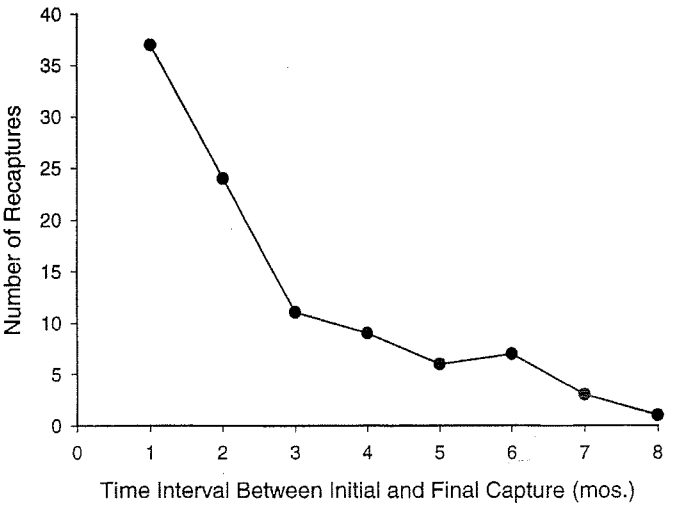

FIG. 3. Time interval between initial marking and final recapture for all *E. wilderae* collected from November 1997 to June 1999.

depend on the individual species, life history stage, and duration of the study period. Any one or a combination of polymer injection, PIT-tagging, and toe-clipping should be suitable for most amphibians. Polymer injection has proven an effective method for long-term marking of amphibian larvae and small-bodied adults, while PIT-tagging and toe-clipping remain effective in marking larger animals. The latter two methods have the added benefit of near invisible marks. In some cases, it may be desirable to use a combination of marking methods on the same individuals. For example, polymer injection could be used to mark individuals throughout the larval stage and, following metamorphosis, PIT-tags or toe-clips could replace the polymer marks. Such a long-term study design would provide valuable life history information and potentially aid amphibian conservation efforts.

Acknowledgments.— We thank Erica Chiao for her artwork and John Hutchens and Wyatt Cross for their comments on earlier versions of this paper. We also thank Sharyn Marks, Chris Beachy, and Don Ashton for providing valuable suggestions. This research was supported by National Science Foundation grant DEB-9629268.

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