

DETERMINING SEX AND LIFE STAGE OF DEL NORTE SALAMANDERS FROM EXTERNAL CUES

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ABSTRACT—Life stage determination for many western plethodontids often requires dissection of the specimen. Availability of reliable external measures that could be applied under field conditions would enhance future studies of the genus *Plethodon*. We examined preserved specimens of the Del Norte Salamander, *Plethodon elongatus*, taken from 11 sites in northwestern California and southwestern Oregon. During the dissections, we recorded several external body characteristics that could prove useful in determining the life stage and gender of individuals. Identification of life stage is possible by using several external measurements. A discriminant function model including snout vent length, weight, head length, and width correctly classified life stage 92% of the time. A tree-based classification analysis identified snout-vent length as a single variable that could correctly classify life stage 90.2% of the time. We also have identified a sexually dimorphic character for adults, vent pattern and coloration, that can reliably distinguish gender at any time of year.

Key words: *Plethodon elongatus*, Del Norte salamander, morphometrics, tree-based classification, gender determination, life stage determination

Past studies of Del Norte salamanders (*Plethodon elongatus*) have focused on identification of the adult life stage. Juveniles and subadults have frequently been lumped together due to a lack of clear characteristics for discriminating these life stages. Snout-vent length (SVL) was the most common discriminator of life stage used in past studies, but in the absence of specific investigations of SVL size ranges by life stage, this yielded a wide variety of size-breaks identified by various authors. Stebbins (1954) identified adults as 61 to 74 mm SVL, Diller and Wallace (1994) identified immature individuals to be 25 to 51 mm SVL and adults as greater than 51 mm. Welsh and Lind (1992) designated individuals as adults if they could be sexed by cloacal examination under anesthesia and were 36 to 63 mm SVL. All individuals that could not be sexed and were 21 to 49 mm SVL were classified as juveniles. Brodie (1970) indicated that sexual maturity was not attained until an individual's SVL reached 55 mm.

Various methods have been used to determine the sex of individuals of many plethodontid species (Stebbins 1954; Brodie 1968, 1970; Welsh and Lind 1992; Diller and Wallace 1994). *Plethodon elongatus* lacks many of the character-

istics that have proven useful to identify gender in other western plethodontid species. Unlike males of the western red-backed salamander (*P. vehiculum*), *P. elongatus* males have no protruding premaxillary teeth or enlarged vent lobes (Ovaska and Gregory 1989). There also seems to be little sexual dimorphism in body size. Male *P. elongatus* also lack the enlarged vent lobes, mental glands, and wider heads of Dunn's salamanders (*P. dunni*; Brodie 1970; LO, pers. obs.). Methods used to determine sex in *P. elongatus* have been limited to determining gravid females by using ova that may be visible through the abdominal wall (Welsh and Lind 1992; Diller and Wallace 1994). During the mating season (fall and spring), sexually active males of *P. elongatus* were distinguished by the presence of enlarged mental glands (Brodie 1968, 1970). Unfortunately, these characteristics were not adequate for distinguishing non-gravid females from subadults or sexually inactive adult males. Although these methods have been used to externally identify life stage and gender of *P. elongatus*, no definitive methods have been developed.

Studies of demography, behavioral responses, and habitat selection can be hampered by a

lack of external cues to the life stage or gender of individuals. In this paper we describe 2 new techniques, confirmed by internal investigation, that may effectively allow for the external sexing of adults and clear discrimination of life stage. Life stage determinations were based on body measurements, while sexual dimorphism was determined using vent pattern and coloration.

METHODS

In 1984 to 1986, *P. elongatus* were collected from 9 localities in Del Norte, Humboldt, and Trinity counties of California and 2 localities in Josephine County, Oregon. The 356 specimens were fixed in 10% formalin solution and stored in 70% ethanol prior to this study. Measurements were taken for each specimen including snout-vent length, total length, head width, head length, and body weight. We recorded descriptions of vent pattern and coloration, enlargement of mental gland, and evidence of a dorsal stripe. Each individual was dissected after measurement of external characteristics. Life stage and gender were determined by dissection using maturity and condition of sex organs as indicators. Juveniles lacked visible sex organs. Subadult females were identified by the presence of small ovaries lacking follicles, coupled with a lack of convolutedness of oviducts. The testes and vasa deferentia of subadult males lacked pigmentation that would indicate if spermatogenesis had ever occurred, and the vasa showed no evidence of enlargement or convolutedness. Adult females were identified by the presence of enlarged, convoluted oviducts and ovarian follicles. Adult males showed pigmented testes and vasa deferentia, indicating prior or present spermatogenesis; enlarged testes and vasa; and convoluted vasa.

Data Analysis

We performed descriptive analyses to review the distributions of the 4 external body measurements (SVL, weight, head width, and head length) by sex and life stage. Comparisons of the means of the measures by gender were performed using unpaired t-tests. In order to develop a classification model for discerning life stage (juvenile, subadult, and adult), a 3-group discriminant function analysis (DFA) also was run on the measurements. A stepwise procedure

was employed to select variables (Affifi and Clark 1990; Burnham and Anderson 1998). Variables were entered into the model if their *P* values for the partial *F* statistic were <0.10 . Once the variables were selected, a linear discriminant function was developed on the basis of those variables. A jackknife procedure was employed to evaluate the classification success of the model (Affifi and Clark 1990; Burnham and Anderson 1998). Cohen's Kappa was computed on the resulting classification matrix to provide a chance-corrected measure of classification success (Titus and others 1984). In this classification procedure, we assumed that composition of our sample represented the true population proportions by life stage; thus, we adjusted prior probabilities of group membership accordingly (set priors proportional; Affifi and Clark 1990; SAS 1990; Huberty 1994; Burnham and Anderson 1998).

Measurement of the 4 external body measures on living, unanesthetized specimens can be difficult to perform in the field without measurement error. Therefore, tree-based classification analysis with stepwise variable selection (Breiman and others 1993) was used to develop a model that would require fewer measurements for accurate life stage identification. This method provides models with greater parsimony than DFA, while maintaining accuracy. Tree-based classification analysis was run unweighted, which was similar to setting priors proportional in the DFA. Tree-based classification analysis attempts to extract the variables that produce the greatest isolation of the groups of interest in the most efficient manner. This method could be likened to the development of a plant key. A series of telling questions are developed that will derive the most logical splits in the data, often going from general to most specific and leading to an unequivocal determination of species identity (Breiman and others 1993). In the case of life stage, measurements from future specimens can be compared directly to the size ranges for each group identified from the tree-based classification analysis. The specimen is readily classified as to life stage without any further mathematical calculations. Thus, this classification can occur in the field at the time of capture.

Males and females were grouped together for all life stage analyses. The level of significance for all statistical analyses was $P = 0.05$.

TABLE 1. Comparisons of body measurements for 356 Del Norte salamanders (*Plethodon elongatus*) from northwestern California and southwestern Oregon. Mean, standard error (in parentheses), and 95% confidence intervals are reported by life stage and sex.

Measure	Subadult			Adult	
	Juvenile n=11	Male n=34	Female n=44	Male n=141	Female n=126
Snout-vent length (mm)	24.63 (1.08) 22.2-27.0	40.03 (0.88) 38.24-41.82	41.23 (0.78) 39.66-42.8	53.19 (0.38) 52.44-53.94	54.39 (0.48) 53.44-55.33
Head length (mm)	5.95 (0.22) 5.47-6.44	9.15 (0.17) 8.79-9.50	9.49 (0.17) 9.14-9.84	12.01 (0.09) 11.82-12.18	12.07 (0.09) 11.88-12.25
Head width (mm)	3.87 (0.14) 3.56-4.19	5.51 (0.13) 5.25-5.78	5.63 (0.09) 5.45-5.82	7.32 (0.08) 7.15-7.48	6.98 (0.08) 6.83-7.13
Weight (g)	0.17 (0.03) 0.10-0.24	0.81 (0.07) 0.68-0.95	0.85 (0.05) 0.76-0.94	1.82 (0.05) 1.72-1.92	1.80 (0.05) 1.70-1.91

All specimens were collected during the course of a prior study in accordance with requirements associated with the State of California Department of Fish and Game Scientific Collectors Permit #8580.

RESULTS

The 356 *P. elongatus* specimens were identified as 11 juveniles, 78 subadults, and 267 adults. The 78 subadults consisted of 34 males and 44 females. There were 141 adult males and 126 adult females. Body measurements for each life stage and gender category are summarized in Table 1.

Sex Determination

There was considerable overlap between adult males and females with regard to the 4 external measures, although significant differences in SVL and head width were identified between sexes (Fig. 1). The overlap in the ranges of adult body measurement demonstrated the inadequacy of a sexing technique based solely on the 4 external measures.

In addition to the 4 external body measurements, presence of the mental gland and ova were used to identify males and females, respectively. Of the 141 adult males examined, 41 had no detectable mental gland. In spring, only 3 of 34 adult females had ova of sufficient diameter to be detectable through the body wall (>2 mm in diameter). In fall, 26 of 92 specimens had ova large enough for accurate determination. Use of these characters would misidentify 29% of the adult males and 77% of the adult females.

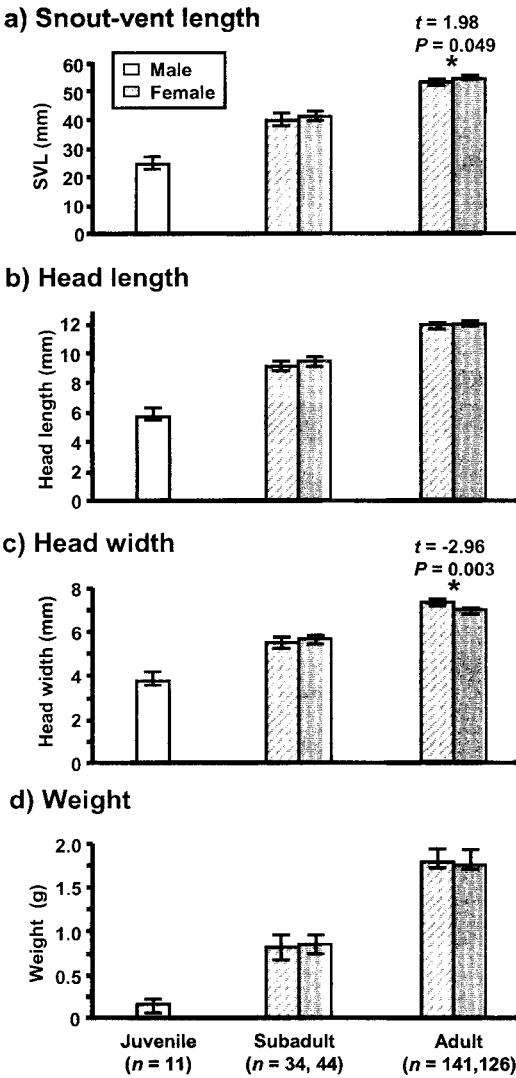
Investigation of vent pattern and coloration yielded more definitive results than the meth-

ods tested above. The vents of adult males were white with smooth to slightly folded margins (Fig. 2). Small to large papillae were evident in a circular patch centered within the depths of the opening. The margins and lining of the vents of adult females were arranged in furrows, often enlarged. The depths of the furrows were white, while the lining between them was counter-shaded dark gray or black. This coloration was visible as a pattern of horizontal lines arranged perpendicular or diagonal to the long axis of the vent (Fig. 2). These characteristics were found to be consistent in all adults dissected. While live subadults would require anesthetization to determine sex by vent characteristics, vent coloration in adult specimens would be clearly evident without magnification.

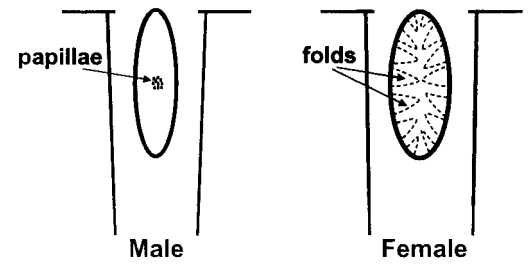
The vents of subadult males were white and predominantly unfurrowed. A small patch of papillae (<0.5 mm in diameter), can be found deep within the vent (Fig. 2). This characteristic is only visible when magnified. In 15 male subadults, no papillae were evident although the vent was smooth. Female subadults also had white vents, but lacked the papillae and showed furrowing of both the interior and margins (Fig. 2). As with the subadult males, the vents of subadult females must be viewed under magnification to confirm gender. The spermatheca was noticeable due to coloration in 19 of 44 subadult females. In those individuals where the spermatheca was not obvious, 4 had striated vents and 12 had clearly folded vents.

Life Stage Determination

Characteristics used in prior studies to discriminate adult *P. elongatus* from other life stag-



Subadult



Adult

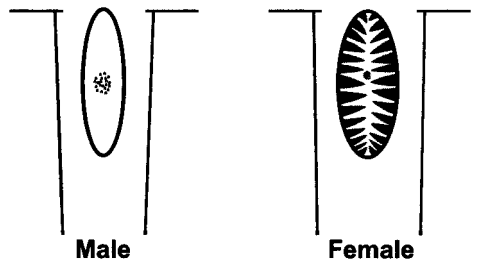


FIGURE 2. Depiction of sex differences in pattern and coloration of cloacal lining in subadult and adult *P. elongatus*.

FIGURE I. Graphical comparisons of body measurements for 356 Del Norte Salamanders, *Plethodon elongatus*, from northwestern California and southwestern Oregon. Bars indicate mean body size and are depicted with a 95% confidence interval for each measure. Measures on adults were compared by gender using Student's t-tests with $\alpha = 0.05$; equal variances were assumed.

es were found to be relatively unreliable for accurately identifying *P. elongatus* adults. Application of those characteristics on our specimens yielded the following: in our sample, 71% of adult *P. elongatus* males showed evidence of a mental gland, and only 23% of the adult females contained ova of sufficient size (>2 mm

diameter) to be detected through the body wall. More reliable characteristics for discrimination were needed.

Thus, we undertook a multivariate analysis of external body measurements that do not vary by season or reproductive status. Four external body measurements were selected for development of a discriminant function model. The linear discriminant function was $1.227(\text{SVL}) - 27.834(\text{weight}) + 4.803(\text{head length}) + 10.217(\text{head width})$. The 1st canonical variate composed primarily of SVL and head length explained 97.96% of the model variability. This model successfully discriminated life stage for 91.85% of the salamanders in our sample (Table 2). Two subadults were incorrectly classified as juveniles, 18 subadults were incorrectly classified as adults, and 9 adults were incorrectly classified as subadults. To determine if the model classification success' was inflated, a Cohen's Kappa test was performed. The Kappa value ($K = 78.35\%$) indicated the model was performing at a lower success rate than reported by the DFA model classification

TABLE 2. Results of a 3-group discriminant function analysis performed to discriminate among life stages using body measurement data from 356 *Plethodon elongatus* captured in northwestern California and southwestern Oregon. Standardized structure coefficients indicate the relative contribution of each variable to the discriminant function.

Step	Variable	F	P	Standardized structure coefficient
1	Snout-vent length	21.71	<0.0001	0.803
2	Weight	17.82	<0.0001	-0.528
3	Head length	7.55	0.0006	0.479
4	Head width	2.53	0.0814	0.184

Wilk's Lambda = 0.2874; $F_{8, 700} = 75.69$; $P = 5-0.001$
 Jackknife success (%) = 91.85; Cohen's Kappa = 0.7835; $P = 5-0.0001$

success, but significantly better than by+chance alone.

The tree-based classification model was composed of a single variable, SVL, as a reliable external physical characteristic for differentiating among *P. elongatus* life stages. The jackknife classification success of this model was 90.2%. The 3 life stages were separated into 3 SVL size groups, juvenile: SVL < 28.45 mm, subadult: SVL 28.45 to 46.74 mm, and adult: SVL \geq 46.75 mm. In this analysis 3 juveniles were misclassified as subadults, 1 subadult was misclassified as a juvenile and 7 were misclassified as adults, and 24 adults were misclassified as subadults. This yielded an overall error rate of 9.84%, similar to the error rate from the linear discriminant function of 8.15%. The similarity in performance between the linear discriminant function and that derived by tree-based classification methods was explained by the high degree of correlation among the 4 body measurements.

DISCUSSION

Gender Determination

The results of this study indicate that the use of vent coloration in *P. elongatus* can provide clear discrimination between the sexes using external means. Gender determination of adults can be achieved in the field, without cooling or anesthetization, by placing individuals into a small plastic bag, which restricts movement and allows a clear view of the vent. Gender determination of subadults requires anesthetization and magnification for proper viewing of indicators. However, this technique does not correctly identify all subadults and

should be used with caution. This tool also allows sexing preserved specimens without dissection.

Characteristics that have been used in past studies are fraught with significant inaccuracy. Other characteristics, such as inter-nares distance (as described by Quinn and Graves 1999), need yet to be investigated for *P. elongatus*. Also, accurately measuring this distance under field conditions may prove difficult without anesthetizing the specimen. Gillette and Peterson (2001) developed the method of candling for sex-determination in red-backed salamanders (*P. cinereus*). This method relies on the presence of dark pigmentation in the testes and vasa. Since breeding condition and dark pigmentation of the testes of male *P. elongatus* vary seasonally, this technique may not be a useful discriminator of gender for this species.

Life Stage Determination

Methods used, by previous investigators to identify life stages for *P. elongatus* were incapable of discriminating juveniles from subadults. Therefore, these 2 life stages were grouped together. In behavioral and mark-recapture studies, as a result of this grouping, behavior of recent hatchlings could not be identified from that of 1- to 3-y-old individuals. Differences in survivorship, food habits, and habitat use likely exist between these life stages. The models developed by this study identified 2 potential methods for assigning life stage to individual *P. elongatus*. There were only marginal differences in error rates between the methodologies, and both could be used in the field. The advantage of the tree-based classification model over the DFA was reduction in the

number of measurements required to determine life stage and a concurrent reduction in opportunities for measurement error. Snout-vent length and body weight are easily performed with accuracy under field conditions, but accurate measurement of head width and length can be difficult to perform on unanesthetized specimens under field lighting conditions.

The development of simple, cost-effective field methods for collecting data on individuals, such as life stage and gender, can enhance a researcher's ability to study the behavior, basic biology, and population ecology of terrestrial salamanders. These data can be used over broad areas or within specific sites to determine rates of population change, to evaluate habitat-use and the importance of habitat features or patch sizes, and to study seasonality, longevity, site fidelity, and the effects of land management on terrestrial salamander populations. Ultimately, field data collected during amphibian studies and monitoring form the basis for evaluating the status of amphibian populations and for making conservation or management decisions concerning those populations.

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