REPRODUCTION AND LIFE HISTORY OF TWO-LINED SALAMANDERS (*EURYCEA* CF. *AQUATICA*) FROM THE UPPER TENNESSEE RIVER VALLEY, USA

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Abstract.—The Two-lined Salamander (*Eurycea bislineata*) species complex is widely distributed across semiaquatic habitats in various ecoregions of the eastern U.S. and Canada of North America. For these reasons, the complex represents a fruitful model for studying the influence of phylogeny and environment on life-history variation. Here, we detail almost 2 y of monthly life-history and demography data from *Eurycea* cf. *aquatica* in the upper Tennessee River Valley, a species and region from which such data were previously unreported. Our principle discoveries are a single-year larval period, with hatching in April or May and metamorphosis the following summer, and the occasional presence of males with nests, which have a mean clutch size of 64 eggs. We further comment on male-male aggression and communal nesting, and we frame our results in the broader ecological and evolutionary context provided by previous studies of the *E. bislineata* species complex.

Key Words.—amphibians; larval growth; mate-guarding; metamorphosis; plethodontid

INTRODUCTION

Life-history variation is influenced by both phylogeny and environment (Wilbur and Collins 1973; Stearns 1983), and organisms that transition between distinct life stages through metamorphosis are particularly compelling models for studying the causes of this variation. Although some amphibians are paedomorphic or direct-developing, many species have a biphasic life history, with aquatic larvae metamorphosing into terrestrial adults. Many amphibians breed in ephemeral wetlands, where abundant food resources enable rapid growth and development (Wilbur and Collins 1973; Williams 1996; Wetzel 1983). Thus, the maintenance of a complex life history has traditionally been explained as a tradeoff between maximizing growth in this aquatic larval stage and maximizing dispersal and reproduction in the terrestrial adult stage (Istock 1967; Wilbur and Collins 1973; Wassersug 1975; Wilbur 1980). Other research has highlighted the importance of additional factors, such as risk of mortality (Werner 1986) and reproductive phenology (Rowe and Ludwig 1991). Spatial or temporal variation in any of these factors can generate a dynamic fitness landscape within or among populations of amphibians (e.g., Berven and Gill 1983; Alford and Harris 1988).

Beachy et al. (2017) argue that larval life history and metamorphosis operate differently in the diverse lungless salamanders (Family Plethodontidae) compared to other lineages of amphibians. Among these differences are: (1) slow growth (Bruce 1980; Voss 1993); (2) relatively long larval periods (e.g., Bruce 1980); and (3) metamorphic timing not predicted by larval growth (e.g., Beachy 1995). Ryan and Bruce (2000) and Bonett

(2016) describe theoretical frameworks and models for studying the mechanisms of life-history variation in plethodontid salamanders.

Among the plethodontids, the greatest variation in biphasic life histories occurs within the Tribe Spelerpini. In this group, larval periods range from approximately 3–60 mo (Anderson and Martino 1966; Bruce 1980), and larval-form paedomorphosis has evolved repeatedly (Bonett et al. 2014). One example of this group is the Two-lined Salamander (*Eurycea bislineata*) species complex, which is widely distributed east of the Mississippi River in the USA and Canada. This species complex currently consists of five named species: the Northern Two-lined Salamander (*E. bislineata*), the Brown-backed Salamander (*E. aquatica*), the Junaluska Salamander (*E. junaluska*), the Blue Ridge Two-lined Salamander (*E. wilderae*), and the Southern Two-lined Salamander (*E. cirrigera*), but molecular systematic studies have suggested both the paraphyly of some of these species and a greater species-level diversity than is reflected by the current taxonomy (Jacobs 1987; Kozak et al. 2006). Members of the *E. bislineata* species complex have a biphasic life history, although larval-form, paedomorphic individuals have been discovered in at least three species (Rose and Bush 1963; McEntire et al. 2014; Carlos Camp, pers. comm.). For most members of this group, oviposition occurs in the winter and spring, and metamorphosis occurs 1–3 y later in the spring and summer (Petranka 1998).

Intrapopulation variation in larval period exists in some members of the *E. bislineata* species complex. For example, in *E.* cf. *wilderae*, Bruce (1985) demonstrated that some larvae metamorphose after approximately one year, while others remain as aquatic larvae for

Figure 1. Male (bottom) and female (top) *Eurycea* cf. *aquatica* with a nest from our study site in Knox County, Tennessee, USA. Visible on the male are its large jaw musculature and scars (indicated with arrows) likely resulting from mate-guarding behavior of a rival male.

an additional year. In the same species, Voss (1993) demonstrated that individuals from first-order streams have a higher frequency of first-year metamorphosis than do individuals from neighboring second- or third-order streams, suggesting a role for fine-scale environmental variation in determining larval period. Both studies noted a poor correlation between growth rates and larval period, suggesting that growth alone does not explain variable larval periods. Beachy (2018) used experimental manipulations to demonstrate that temperature, but not growth rate, has a strong effect on the timing of metamorphosis in *E.* cf. *wilderae*, providing a mechanism to explain variation in larval period among stream orders. Other species of *Eurycea* that breed in more temporary aquatic habitats have much shorter larval periods, providing evidence that impermanency of larval habitat selects for shorter larval period (Bruce 1982a). This may not be an important factor, however, in predicting larval period in previously studied populations of the *E. bislineata* species complex, which primarily breed in hydrologically stable habitats (Bruce 1982a). Marshall (2000) underscored a potential alternative mechanism for these patterns by demonstrating a strong positive correlation between date of oviposition and larval period across the *E. bislineata* species complex, with early oviposition dates predictive of shorter larval periods. Thus, the length of the larval period in the *E. bislineata* appears to be partially determined by environment and behavior. In contrast, Bruce (1982a) showed that sympatric *E.* cf. *wilderae* and *E. junaluska* oviposit at approximately the same time

but have different average larval periods, suggesting a role for phylogeny in addition to environment.

Members of the *E. bislineata* species complex typically oviposit in lotic nesting sites, although geographic and population-specific variation in nest-site selection has been described (e.g., Bahret 1996; Guy et al. 2004). Females typically attach clutches of approximately 10–150 eggs to the underside of stones, but eggs may instead be scattered among roots or leaf packs (Wood 1953; Bahret 1996; Petranka 1998). As in many other plethodontid salamanders, females in this group are often found in attendance of their clutch, although the forms of parental care they provide are not as well studied as in other species (e.g., Forester 1979). Unlike other plethodontid salamanders, males in the *E. bislineata* species complex are also sometimes found with nests, although this is apparently only true for forms with guarding male reproductive tactics (e.g., Bruce 1982b; Graham et al. 2010; Hutton and Pierson 2017; Pierson and Paulson 2017; Pierson et al. 2019). In brief, this reproductive tactic includes morphological traits (i.e., seasonally hypertrophied jaw musculature) correlated with behavior (i.e., mate-guarding) that occurs at nesting sites.

Here, we describe a study of the reproduction and life history of one member of the *E. bislineata* species complex from a population in the upper Tennessee River Valley of the USA (Fig. 1). This population is a member of the clade that includes *E. aquatica* and *E. junaluska* and is most similar in gross morphology to *E. aquatica*, but a thorough analysis of gene flow among

Figure 2. An outline map showing Tennessee, USA. The dark green polygon represents the upper Tennessee River drainage, part of which is inhabited by *Eurycea* cf. *aquatica*. The black dot represents our study site at Forks of the River Wildlife Management Area.

putative species in this clade has not been completed (Jacobs et al. 1987; Kozak et al. 2006; Timpe et al. 2009; Graham, pers. comm.). Thus, we refer to this population as *Eurycea* cf. *aquatica* for the remainder of this manuscript. We describe oviposition, larval growth, and metamorphosis and place this study in the context of broader research on the life history of the *E. bislineata* species complex.

Materials and Methods

We conducted this study in a small, partially spring-fed limestone creek in Forks of the River Wildlife Management Area in Knox County, Tennessee, USA (35.9384°N, 83.8472°W; WGS 84; Fig. 2). This stream flows directly into the French Broad River shortly before it joins the Holston River to form the Tennessee River, placing it in the larger upper Tennessee River drainage. It is partially ephemeral, and during the summer, large stretches of the stream dry entirely and are devoid of surface-dwelling larval salamanders (pers. obs.), perhaps in contrast to the mostly stable groundwater spring habitats of *E. aquatica* elsewhere.

We primarily sampled from stretches of this stream that held water year-round. Beginning in August 2016 and continuing through June 2018, we conducted approximately monthly sampling for a total of 21 mo. Each month, we used small dipnets and searched by hand to catch up to 50 larval *Eurycea* cf. *aquatica*, searched underneath rocks along and near the streambank for metamorphosed individuals, and in the breeding season, searched under submerged rocks for nests. For all larvae, we used a metric ruler to measure snout-vent length (SVL) to the nearest millimeter. For all metamorphosed animals, we also measured head-width (HW) to the nearest millimeter and noted the presence and location of any scars suggestive of bites from other salamanders. To estimate measurement error, one of us (TWP) measured SVL five times each for five larvae and measured SVL and HW five times each for five adults. We calculated relative error as the absolute value of the difference between a measurement and the mean of all five measurements, divided by the mean of all five measurements. We determined the sex of metamorphosed animals by the presence

of mental glands in males and presence of ova visible through the abdominal skin in females; all animals that had neither of these characteristics were omitted from sex-dependent analyses. For all nests, we took a photograph to document the number of eggs and noted whether we found any adults in close proximity. Following measurements and photographs in the field, we immediately released all animals back at the point of capture.

We conducted all analyses in R v3.5.1 (R Core Team 2018). To quantify differences in relative head size, we calculated the ratio between HW/SVL separately for males and females, tested for the normality of these distributions using Shapiro-Wilk tests (alpha = 0.05), and compared these two groups using a *t*-test (alpha = 0.05). To evaluate larval period, we plotted larval SVL as a SinaPlot using the sinaplot package (Sidiropoulos et al. 2017) and visually examined the monthly distributions for evidence of unimodality or multimodality (*sensu* Bruce 1988). Within each cohort, we calculated mean monthly growth rates using the change in mean SVL and the number of days between sampling periods; for this calculation, we excluded periods during which the metamorphosis of some animals (and thus the removal of those samples from the larval population) caused the mean larval SVL to decrease (e.g., between April and May 2017). We calculated the distribution of metamorph SVL using all animals in the process of or having just completed metamorphosis.

Results

We recorded 877 captures of larvae (mean = 41.8 per survey ± [SD] 14.1; range, 5–50) and 98 captures of metamorphosed animals in our 21 surveys. Because we did not mark or remove animals, we cannot determine the frequency of recaptures between sampling periods. We estimated low relative error for larval SVL (mean = 0.02 ± 0.02; range, 0.00–0.07), adult SVL (mean = 0.01 ± 0.01; range, 0.00–0.03), and adult HW (mean = 0.02 ± 0.03; range, 0.00–0.11) measurements. Of the 98 metamorphosed individuals, we could confidently assign a sex to 76. The distributions of female and male HW/SVL ratios were not significantly different from normal ($W = 0.98$, $P = 0.760$; $W = 0.96$, $P = 0.190$). Adult males had larger HW/SVL ratios (mean = 0.16 ± 0.02; range, 0.13–0.20, n = 41) than females (mean = 0.14 ± 0.01; range, 0.11–0.17, n = 35), and this difference was significant ($t = 5.609$, df = 72.997, $P < 0.001$; Fig. 3). We only found sexually mature adults in the stream channel and under rocks on the stream bank between October and April. We found scars and wounds suggestive of intraspecific biting behavior in 10 males and one female; with the exception of one male from January 2018, we found all of these individuals in
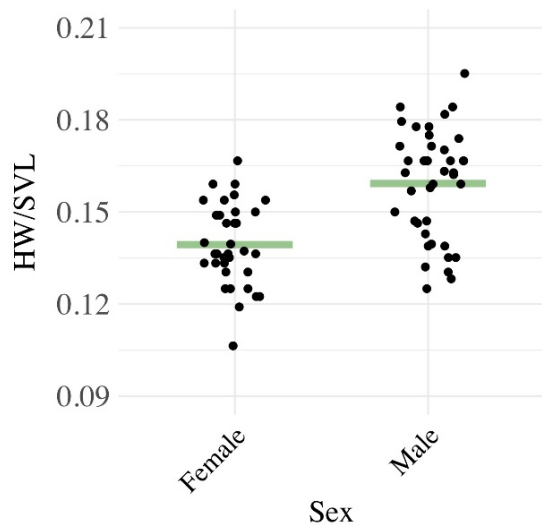
FIGURE 3. SinaPlots of head width (HW): snout-vent length (SVL) ratios for adult female and adult male *Eurycea* cf. *aquatica*, from Knox County, Tennessee, USA. Each point represents one salamander; thick green lines represent the means of each group.

March and April. In general, these scars and wounds were concentrated towards the base of the tail and posterior third of the dorsum of the salamanders.

We discovered 37 nests with an average clutch size of 64 eggs (mean = 63.8 ± 21.1 eggs; range, 22–104). In 2017, we found two nests on 4 February, one nest on 10 March, and 11 nests on 7 April. In 2018, we found one nest on 21 January, 16 nests on 8 March, and six nests on 14 April. Of the 37 nests, 13 nests had no adult

present, 21 nests had only a female present, one nest had only a male present, and two nests had both a male and a female present.

We first found hatchlings on 1 May 2017 and 14 April 2018, and we found no nests later than 14 April, suggesting a hatching period from mid to late April. Plotting larval SVL revealed a unimodal distribution in most months, with the exception of a short period of overlap between cohorts following hatching (Fig. 4). These data indicate a single-year larval period. We calculated a mean larval growth rate of 0.07 mm/day (mean = 0.07 ± 0.05 mm/day; range, -0.1–0.15, n = 19 mo). We saw the first morphological indications of metamorphosis on 10 March 2017 and 14 April 2018 but did not find fully-metamorphosed individuals until 14 June 2017 and 15 May 2018. Most metamorphosis occurs between late April and mid-June, although some larvae did not metamorphose until as late as July. Animals in the process of metamorphosis or having just completed metamorphosis measured between 27 and 41 mm SVL (mean = 33.1 ± 3.2 mm; n = 49).

DISCUSSION

The seasonal activity of adult *Eurycea* cf. *aquatica* is consistent with descriptions of its closest relatives, *E. aquatica* and *E. junaluska* (Rose and Bush 1963; Graham et al. 2013; Bruce 1982b; Sever 1983). Likewise, the greater relative head width in males has been thoroughly detailed in *E. aquatica* (Alcorn et al. 2013; Graham et al. 2013) and demonstrated to be correlated with mate-guarding behavior across the *E. bislineata* species
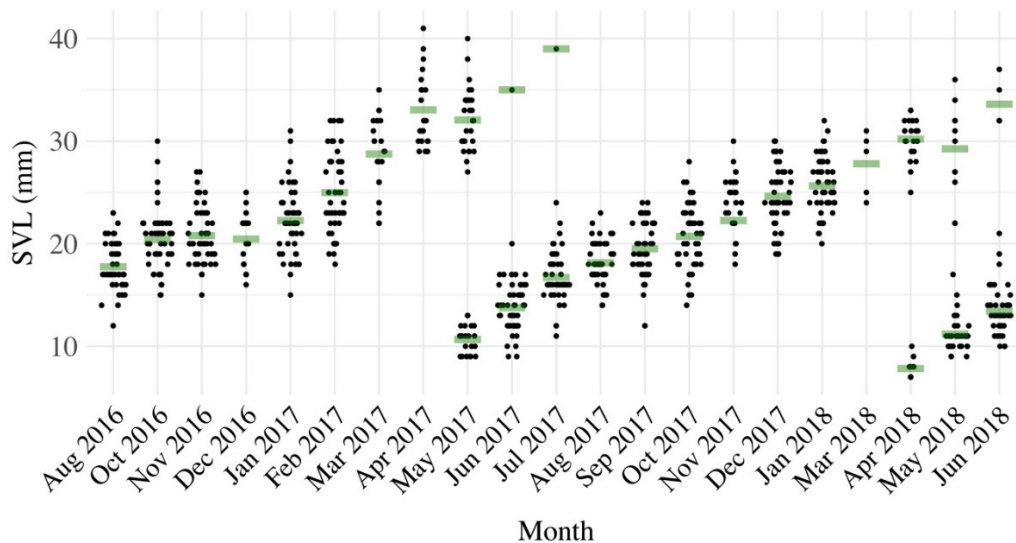

FIGURE 4. SinaPlots of larval snout-vent length (SVL) of *Eurycea* cf. *aquatica* from August 2016 to June 2018 from Knox County, Tennessee, USA, demonstrating overlapping, single-year larval periods. Each point represents one salamander. Thick green lines represent the monthly means of each cohort.

complex (Graham et al. 2010; Deitloff et al. 2014; Pierson et al. 2019). The timing and prevalence of the scars we observed are also consistent with this behavior and match those described in previous studies of this group (Deitloff et al. 2014; Pierson et al. 2019). Deitloff et al. (2014) suggested that mate-guarding behavior and its accompanying morphology may be an adaptation of *E. aquatica* to its restrictive habitat requirement of freshwater springs. Although this may play a role in the especially exaggerated sexual dimorphism in this species, this reproductive tactic is better predicted by phylogeny (Pierson et al. 2019), and its evolutionary history is likely more complicated.

We observed a mean clutch size of 64 eggs, which is similar to values reported for *E. aquatica* (Rose and Bush 1963; Graham et al. 2010; Jones 1980) and larger than values reported for *E. junaluska* (Bruce 1982b; Ryan 1998). In a previous field season at this same location, we discovered a nest of 154 eggs (Pierson and Paulson 2017). Possibly, this large nest consisted of eggs deposited by multiple females, but Jones (1980) reported 153 enlarged ova in a single female *E. aquatica*. We repeatedly found multiple nests under the same rock or under stacked, adjacent rocks, similar to observations of communal nesting in other members of this group (e.g., Wood 1953; Baumann and Huels 1982; Jakubanis et al. 2008; LeGros 2011; Ferguson et al. 2014). Three of the nests we observed had males present, also similar to observations in other members of this group (Bruce 1982b; Graham et al. 2010; Hutton and Pierson 2017; Pierson and Paulson 2017; Pierson et al. 2019). Notably, these observations of communal nesting and the presence of males with nests all come from populations of (or in polymorphic populations, from individual) guarding males (Pierson et al. 2019). Although common in frogs, male parental care is rare in salamanders and is documented only in the few groups with external fertilization (e.g., Cryptobranchidae and Hynobiidae; Kawamichi and Ueda 1998; Usuda 1997). Nussbaum (1985, 2003) argues that the sex that provides parental care is likely determined by the sex that selects the site of oviposition. Because plethodontid salamanders have internal fertilization and a courtship period that is often separated from oviposition by weeks or months, there is little opportunity for male selection of nest sites in most species. Males of the *E. bislineata* species complex with the guarding reproductive tactic may be an exception. The presence of these males with nests may be incidental and merely a byproduct of mate-guarding near nesting sites, but repeated observations of the same male with the same nest (Graham et al. 2010) suggest the possibility of biparental attendance of nests. Careful observations of the behaviors of male *Eurycea*

at nesting sites, especially in the laboratory, will help resolve these questions.

Our results clearly demonstrate a single-year larval period for this population. The larval period for *E. aquatica* is not well-described, but our results contrast with those from *E. junaluska*, which typically has a multi-year larval period (Ryan 1998). These differences are consistent with the strong correlation between date of oviposition and larval period across the *E. bislineata* species complex (Marshall 2000), as *E.* cf. *aquatica* in our study exhibit early oviposition and a short larval period; whereas, *E. junaluska* exhibit later oviposition and a longer larval period. Alternatively, the single-year larval period in this population may be the result of the hydrological instability of the study stream, even though our sampling was focused in stretches of the stream that held water year-round. Replication of this study at other streams with variable hydrological stability would help to resolve this question.

We estimated an average growth rate of 0.07 mm/day, which is an order of magnitude higher than the slowest-growing members of the *E. bislineata* species complex (e.g., Johnson and Wallace 2002). In the cohort that hatched in 2017, we observed a relatively linear growth pattern, similar to that which was documented in other studies of this group (e.g., Beachy 1997; Johnson and Wallace 2002). In contrast, the first cohort we measured appeared to show slowed growth from October to December 2016 and expedited growth in the months following. The beginning of this period was unusually hot and dry (e.g., simultaneous with the nearby wildfires in Gatlinburg) and was followed by a period of unusually high rainfall (Lawrimore, J. 2016. Global Summary of the Month, Version 1.0. Knox County, TN, USA. NOAA National Centers for Environmental Information. DOI:10.7289/V5QV3JJ5. Available from https://ezid.cdlib.org/ [Accessed 28 February 2019]), and it is possible that this anomalous weather contributed to the variable growth rates in our sample. Metamorphosis in all populations of the *E. bislineata* species complex, including the one in the present study, is concentrated in the late spring and summer. We observed SVL at metamorphosis ranging from 27–41 mm, which is similar to sizes reported from *E. aquatica* (Rose and Bush 1963) and slightly smaller than those from *E. junaluska* (Ryan 1998).

The *E. bislineata* species complex inhabits diverse habitats across its wide distribution, and fundamental life-history parameters vary among and within populations. Larval period appears to be predicted by a combination of phylogeny, environment, and behavior, with longer larval periods typical of species: (1) in the northern clade (Petranka 1998; Kozak et al.

2006; Timpe et al. 2009; Pierson et al. 2019); (2) with late oviposition (Marshall 2000); and (3) developing at colder temperatures (Beachy 2018). One member of this northern clade is *E. junaluska*, which lives in cooler, montane streams and typically has a multi-year larval period. Nearby and at the same latitude, *E.* cf. *aquatica* can be found in springs and streams in the Tennessee River Valley. Here, we demonstrate that in one such stream, this species has relatively rapid growth and a short larval period, but we expect that at other sites with different temperature regimes, it may have a multi-year larval period. The interacting (and often, confounded) effects of phylogeny, environment, and behavior are difficult to disentangle in field studies such as this one, and additional manipulative experiments (e.g., Beachy 2018) should be used to better understand the proximate and ultimate drivers of life-history variation in this group.

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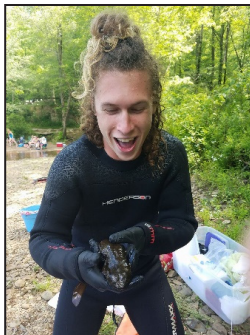
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