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Courtship Behaviors of Three-Lined Salamanders (*Eurycea guttolineata*)

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Abstract.—The courtship behaviors of plethodontid salamanders have long been of interest to biologists studying topics such as the evolution of complex phenotypes and the origin of reproductive isolation. However, the paucity of basic natural history data for some salamanders — including some rather widely distributed and common species — creates limitations for comparative research. We herein provide the first detailed account of courtship behaviors in Three-Lined Salamanders (*Eurycea guttolineata*). We staged trials with wild-collected adults from near Atlanta, Georgia, and observed courtship behaviors in seven of eight trials. Of those seven trials, all resulted in spermatophore deposition and five resulted in successful spermatophore transfer. Overall, the courtship behaviors of *E. guttolineata* are similar to those of other species in the genus and include "snapping" pheromone delivery — a behavior not previously observed in the closest relatives of *E. guttolineata*. We provide an ethogram and preliminary interpretations of these behaviors and encourage others to collect more basic natural history observations.

Lungless salamanders (Family Plethodontidae) engage in ritualized courtship preceding the external transfer of a spermatophore, and studies of these courtship behaviors have proven fruitful for studying broader biological themes like the evolution of reproductive isolation (e.g., Houck et al. 1988; Mead and Verrell 2002; Kozak 2003; Faddis et al. 2024). However, comparative studies of these complex phenotypes (e.g., Sever et al. 2016) can be hindered by the absence of descriptions for many taxa — including common and widely distributed species. Thus, the gradual accumulation of this basic natural history information from understudied taxa (e.g., Pierson et al. 2017; Marvin 2024) can provide an important foundation for future research.

Three-Lined Salamanders (*Eurycea guttolineata*) are relatively large brook salamanders found primarily in the Piedmont and Coastal Plains physiographic regions of the southeastern United States. Despite being rather common and widely distributed, little is known about their reproductive natural history. Oviposition likely occurs from late fall through winter (Gordon 1953; Bruce 1970, 1982; Marshall 1999; Freeman and Bruce 2001), with courtship presumably occurring in the preceding months when reproductively mature adults show increased surface activity (Enge 1998; Marshall 1999). In this courtship season, male *E. guttolineata* exhibit mental glands and protruding premaxillary teeth (Dunn 1926), used to produce and deliver courtship pheromones (Kiemnec-Tyburczy et al. 2009). Noble and Brady (1930) acknowledged having observed courtship in *E. guttolineata*, but they provided no detailed descriptions of courtship behaviors, and none have been reported elsewhere (Petranka 1998). We herein provide the first detailed description of the courtship behaviors of *E. guttolineata* by staging trials with animals collected near Atlanta, Georgia, USA.

Methods

Field collections and captive maintenance.—On 17 September 2023, we collected two adult female and four adult male *Eurycea guttolineata* from a large river floodplain in Big Creek Park (34.03479, –84.30179), which is located in the Piedmont physiographic province in Fulton County, Georgia. We identified females by the presence of yolked ova visible through the venter and males by the presence of seasonal secondary sexual characteristics (i.e., elongate cirri and a mental gland). We housed salamanders in a dedicated laboratory at Kennesaw State University, maintained this room near 19 °C, and used an automatic timer to control a single incandescent lamp in the room, which we established on a reverse daylight schedule approximating the natural photoperiod (i.e., on at 2000 h and off at 0800 h). We housed each salamander individually as described in Faddis et al. (2024), and we fed salamanders a diet of small crickets. We allowed salamanders to acclimate for eight days prior to the onset of courtship trials.

Courtship trials.—Between 25 September and 12 October 2023, we staged eight courtship trials, sequentially pairing each female with each male and separating consecutive trials by at least four days. We began trials between 1157 h and

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1316 h (i.e., approximately 4 and 5 h after artificial dusk), and we illuminated the room with faint red light to facilitate video recording. We conducted trials in modified housing containers, which we lined with moist paper towels, and we video-recorded each trial with a HERO 3 GoPro camera. For each trial, we simultaneously placed the male and the female into the enclosure, started the recording, and left the room for 159–193 min. After the trial, we checked the female's cloaca for the presence of a spermatophore, which is visible for at least 15–24 h after transfer (Reagan 1984; Verrell 1991), and returned each salamander to its respective home enclosure.

Data collection and analysis.—We reviewed videos and created a catalog of behaviors, which we modeled after those from other relevant studies of plethodontid salamanders (Arnold 1972; Kozak 2003; Marvin 2024). Using this catalog, we noted each instance of a behavior in each video. We then used the package igraph v2.1.2 (Csardi and Nepusz 2006) in R v4.4.2 (R Core Team 2024) to create and plot a directed graph showing the temporal organization of behaviors.

Our primary goal was to provide a qualitative description of courtship behaviors, and because we had both a very low sample size and an inability to correct for individual effects (i.e., from the reuse of the same animals in multiple trials), we did not conduct formal statistical analyses. However, we did explore preliminary differences in behaviors between successful and unsuccessful courtship encounters. We considered an "encounter" to be a series of courtship behaviors that led to spermatophore deposition; using this definition, a single trial could include multiple encounters. Thus, we excluded the one trial with no courtship behaviors and compared encounters that resulted in spermatophore transfer (n = 5 encounters in 5 trials) to those that did not (n = 3 encounters in 2 trials). We created boxplots to explore preliminary differences in six behaviors: the number of times the male approached the female's head, the number of times the male approached the female's body, the number of nudges, the number of times the male initiated head contact, the number of snaps, and the cumulative duration of tail-straddling walk. We organized data and generated all plots in R v4.4.2 (R Core Team 2024).

Results

We observed courtship behaviors in seven of the eight trials. Of those seven trials, all resulted in spermatophore deposition and five resulted in successful spermatophore transfer (as indicated by the presence of a spermatophore in the female's cloaca). In the two trials that included courtship behavior but no spermatophore transfer, we found one and two full spermatophores on the substrate, respectively. From the seven trials that included courtship, we created a catalogue of behaviors.

Catalog of courtship behaviors.—Behaviors performed by females were: *Move away*: moves away from the male and *head contact*: using the head to initiate contact with the head of the male. The latter behavior was sometimes reciprocated (i.e., similar to "mutual cheek rubbing"; Reagan 1984).

Behaviors performed by males were: *Approach head*: approaches the female near the head, typically taking a perpendicular course and blocking her path forward; *approach body*: approaches the female away from the head, taking either a parallel or perpendicular course and ending at the female's body or tail; *nudge*: using the head to make contact with the tail or body of the female; *head contact*: using the head to initiate contact with the head of the female (when performed by males, this contact is sometimes directed toward the chin of the female; i.e., similar to "lifting"; Arnold 1972); and *snap*: pulling the snout forcefully and suddenly across the skin of the female, often throwing the two salamanders away from each other.

Behaviors performed together by both sexes were: *Tail-straddling walk (TSW)*: male moves forward while undulating tail and the female follows while straddling the male's tail; *stationary with tail undulation*: male ceases movement forward, keeps body stationary, and continues undulating tail; *position over spermatophore*: male flexes tail, holding it at a 45–90° angle relative to the side of his body, and walks forward; the female follows, and both stop when the female is over the spermatophore; and *stationary with tail flexed*: male flexes tail and rhythmically extends and flexes hindlegs, pushing the dorsal tail base into the female's snout.

Temporal organization of courtship behavior.—The temporal organization of many courtship behaviors was relatively consistent across trials (Fig. 1). Typically, the male moved toward the female's head or body. As the male approached, he sometimes nudged and only rarely snapped. When the male approached the female's head, he sometimes made head contact. In most instances, the female responded to both of these approaches by moving away from the male, after which the male usually approached again. Eventually, after the male approached the female's head, the two would make reciprocal head contact. The male often appeared to lift his head beneath the female's chin, and the female pushed her head into the cheeks or top of the male's head (i.e., approximating "lifting" [Arnold 1972] and "mutual cheek rubbing" [Reagan 1984]). The two salamanders then began tail-straddling walk (TSW) immediately after reciprocal head contact. In TSW, the male undulated his tail and walked slowly. In some cases, the male turned backward and snapped — a sudden and forceful behavior that physically separated the two. The salamanders then resumed TSW. Eventually, the male stopped walking and continued undulating his tail, during which he deposited a spermatophore, although we could not observe this directly in our videos. The male then flexed his tail and placed it to the side, walking forward with the female following and stopping when the female was positioned approximately on top of the spermatophore. The male kept his tail

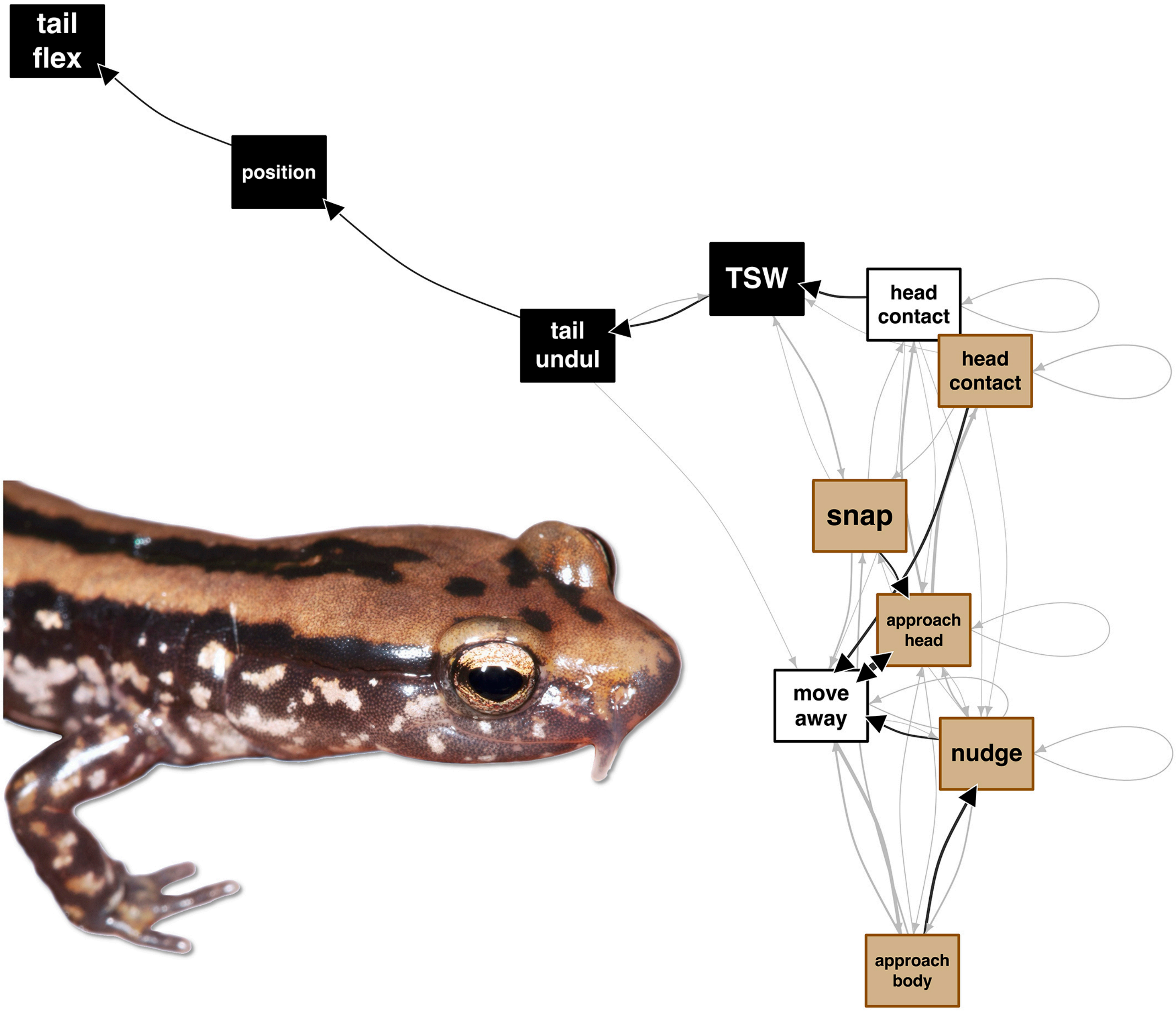

Figure 1. Composite diagrammatic ethogram of courtship behaviors in Three-Lined Salamanders (*Eurycea guttolineata*) based on eight separate trials. Line weight is proportional to the relative frequency of transitions. Black arrows show the most common transition from each behavior, and gray arrows show all other transitions. Tan boxes show male behaviors, white boxes show female behaviors, and black boxes show behaviors employed by both sexes. TSW = tail-straddling walk; tail undul = stationary with tail undulation; position = position over spermatophore; tail flex = stationary with tail flexed. The inset image shows an adult male *Eurycea guttolineata*. Photograph by Todd W. Pierson.

flexed and engaged in repeated extensions and flexions of his hindlimbs — pushing his dorsal tail into the female's chin. During this stage the female locates and picks up the spermatophore, but as with spermatophore deposition, we could not directly observe this behavior from our videos.

Preliminary differences between successful and unsuccessful courtship encounters.—We observed a greater number or greater duration of all six courtship behaviors in successful courtship encounters than in unsuccessful encounters (Fig. 2), but we did not conduct any formal statistical comparisons, and these results should be interpreted with caution. The number of times the male approached the female's head was higher in successful encounters (median = 17; range = 3–25) than in unsuccessful encounters (median = 9; range = 7–12). The number of times the male approached the female's body was higher in successful encounters (median = 6; range = 0–12) than in unsuccessful encounters (median = 1; range = 1–2). The number of times the male nudged was higher in successful encounters (median = 7; range = 0–8) than in unsuccessful encounters (median = 0; range = 0–1). The number of times the male made head contact was higher in successful encounters (median = 3; range = 1–6) than in unsuccessful encounters (median = 2; range = 2–5). The number of times the male snapped was higher in successful

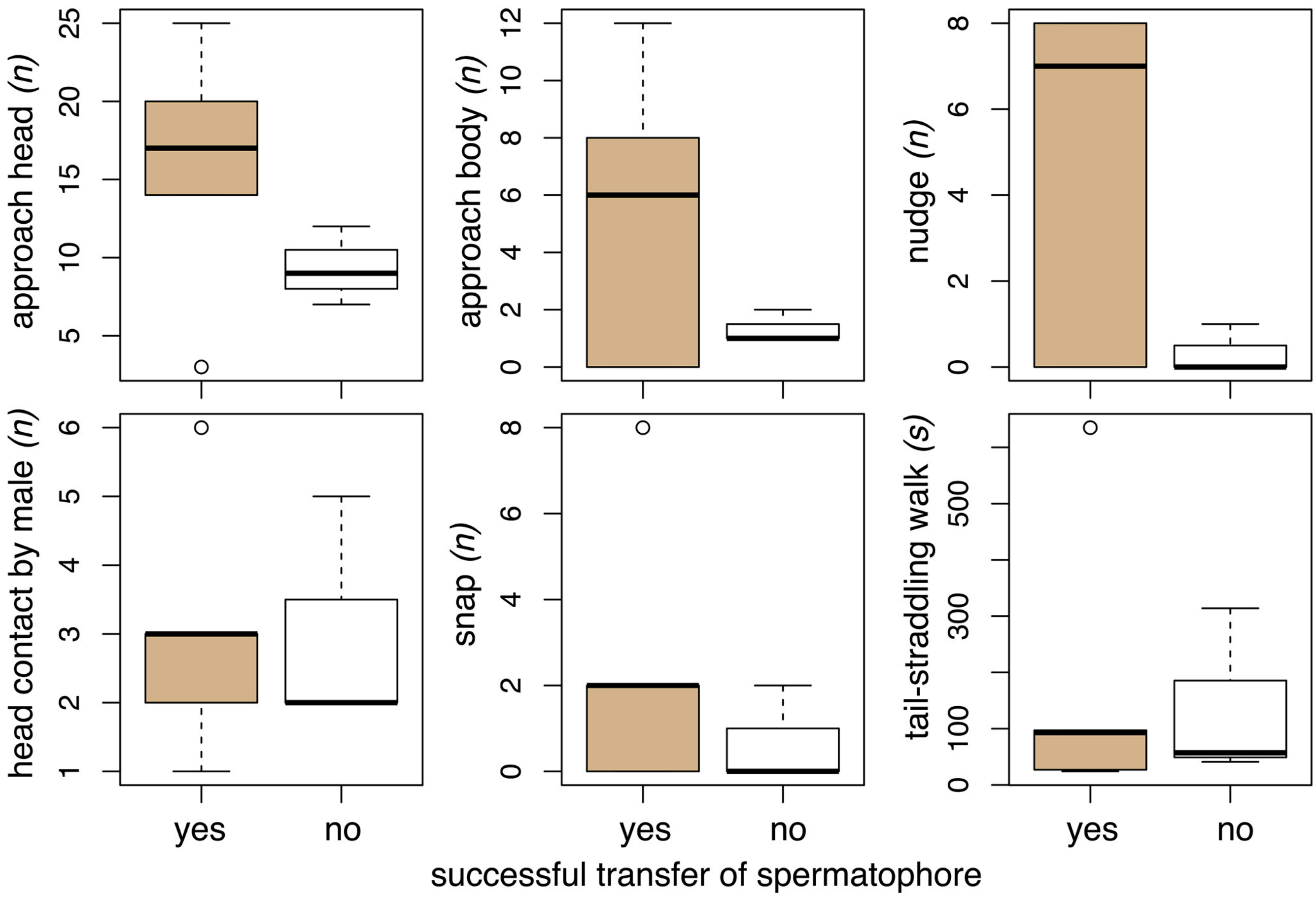

Figure 2. Boxplots showing preliminary comparisons of courtship behaviors in Three-Lined Salamanders (*Eurycea guttolineata*) between encounters that led to successful spermatophore transfer (*n* = 5 encounters in 5 trials) and those that did not (*n* = 3 encounters in 2 trials).

encounters (median = 2; range = 0–8) than in unsuccessful encounters (median = 0; range = 0–2). Finally, the total time spent in tail-straddling walk was higher in successful encounters (median = 93 s; range = 24–635 s) than in unsuccessful encounters (median = 57 s; range = 41–314 s).

Discussion

Overall, the courtship behaviors that we documented in *Eurycea guttolineata* were very similar to those in other species of biphasic *Eurycea*, including behaviors also witnessed in *E. bislineata* (Noble 1929; Arnold 1972), *E. cirrigera* (Kozak 2003), *E.* cf. *wilderae* (Reagan 1984; Kozak 2003; Faddis et al. 2024), and *E. lucifuga* (Organ 1968).

Perhaps our most notable observations are those of snapping behavior. This is well-documented in members of the *E. bislineata* species complex (Arnold 1972; Kozak 2003), but missing from descriptions of courtship in *E. lucifuga* (Organ 1968) and a (very limited) description of *E. longicauda* (Cooper 1960) — the two closest relatives of *E. guttolineata*. In their phylogenetic reconstruction, Sever et al. (2016) considered this behavior to be absent in *E. lucifuga* and *E. longicauda*. These two species also reportedly lack the fan-shaped mental gland characteristic of the *E. bislineata* species complex, instead exhibiting a rounder "large pad" (Sever 1976; Sever et al. 2016; Alvino and Siegel 2017) — a description consistent with our observations of mental glands of *E. guttolineata*. We also confirmed the presence of enlarged premaxillary teeth piercing the upper lip in male *E. guttolineata* (Dunn 1926), and these teeth often are associated with snapping (Arnold 1972; Houck and Sever 1994).

We observed snapping in four of the eight trials. Like previous reports of snapping in *Eurycea*, we noted this behavior both while the male was facing the female in the "persuasion phase" (i.e., the "preliminary behaviors" before or between instances of TSW; Staub et al. 2020), and also during TSW (Arnold 1972; Reagan 1984; Kozak 2003). In the latter case, the male turned around and snapped, which temporarily threw the two apart and interrupted TSW. That we did not observe any pulling behavior is notable; pulling is similar to snapping, but slower and less forceful, and occurs before TSW in other species of *Eurycea* (Arnold 1972; Kozak 2003).

Our primary goal was to provide a descriptive natural history of courtship behaviors with a very small sample size and using the same individual males and females in multiple trials. These limitations precluded a more thorough statistical assessment of the behaviors that predicted successful spermatophore transfer (e.g., Marvin 2024), but we elected instead to present some preliminary summaries of behaviors in successful versus unsuccessful courtship encounters. In general, we observed a greater number or greater duration of each of these behaviors in successful courtship encounters.

We note that all three unsuccessful courtship encounters involved the same male (Eg006), which deposited a total of three spermatophores across the two trials. In the trial with the first female (Eg001), the pair appeared to undergo typical behaviors through spermatophore deposition and positioning, but the female disengaged and moved away while the male was stationary with his tail flexed. In the trial with the second female (Eg002), the pair engaged in tail-straddling walk (TSW), and the male began spermatophore deposition while stationary and undulating his tail. However, the female quickly moved away — ending this encounter. The male continued to pursue the female, and approximately two hours later, the pair resumed TSW. This time, the male appeared to stop TSW twice and remain stationary with tail undulation before resuming TSW. In the third instance of this stationary tail undulation, he appeared to deposit a spermatophore; the pair positioned over the spermatophore, and the male began rhythmic hindlimb flexion and extension while remaining stationary with his tail flexed. However, the female moved away during this stage and ended the encounter. Although interesting that this male had particularly low courtship success, we caution against the overinterpretation of our small dataset.

We acknowledge several additional limitations of our study design. First, the manner in which we recorded the trials (i.e., from overhead) prevented us from clearly documenting some behaviors. We were not convinced that we could conclusively identify nose-tapping behavior from our videos, and the angle of the recording prevented us from clearly documenting more specific female behaviors associated with spermatophore transfer (e.g., lowering of the vent and positioning over the spermatophore cap). Additionally, the salamanders occasionally climbed the walls of the plastic enclosures and out of view of the camera. As a consequence, some of our recordings are missing portions of courtship sequences.

In summary, we present the first detailed descriptions of courtship behaviors in *Eurycea guttolineata*. We hope that our study can illustrate the ease with which these basic natural history data can be collected, and we encourage further studies gathering similar observations to collectively inform future comparative investigations.

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