Histological and Osteological Variation between Alternative Reproductive Tactics in Two-Lined Salamanders

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ABSTRACT: In some populations of the Two-Lined Salamander (*Eurycea bislineata*) species complex, males exhibit two alternative reproductive tactics: "searching" and "guarding." Searching males have secondary sexual characters—including a large, pheromone-producing mental gland—used in terrestrial courtship, whereas guarding males have hypertrophied jaw musculature used in mate-guarding behavior near aquatic nesting sites. Although this polymorphism occurs in at least four evolutionarily distinct lineages, previous histological studies have only focused on soft-tissue differences in one of these species and no studies have evaluated osteological differences between alternative reproductive tactics. Herein, we present new histological and osteological data from three evolutionarily distinct lineages within the *E. bislineata* species complex. We confirmed that traditional mental glands are restricted to searching males and that caudal courtship glands are present in both male phenotypes, but not in females. We also found variation in other skin glands that warrants further investigation. Cleared and stained specimens and microcomputed tomography data both revealed substantial osteological differences in skull morphology. Compared with searching males, guarding males have increased skull ossification, fewer and larger teeth, and more pronounced otic and squamosal crests. Together, these data expand our knowledge of morphological differences between alternative reproductive tactics, and we discuss the implications of our results for hypotheses regarding phenotypic plasticity throughout the lifetime of an individual male.

Key words: Courtship; *Eurycea*; Mental gland; Microcomputed tomography; Secondary sexual characteristics; Skull morphology

In lungless salamanders of the family Plethodontidae, males of most species are recognizable by a suite of secondary sexual characters present during the courtship season. The most prominent of these features is the mental gland, a cluster of submandibular exocrine glands that hypertrophy seasonally (Sever 1975; Rollmann et al. 1999; Wilburn and Feldhoff 2019). This mental gland is used to deliver courtship pheromones (Sever 1975; Houck and Arnold 2003) that increase female receptivity to courtship, decrease courtship time, and increase the probability of spermatophore deposition and insemination (Houck and Reagan 1990; Rollmann et al. 1999, 2003). Although the mental gland is considered a synapomorphy of plethodontids, its structure and function vary tremendously across species and it has been independently lost in several groups (Houck and Sever 1994; Sever et al. 2016a).

Other secondary sexual characters are perhaps less obvious. Male plethodontid salamanders also exhibit caudal courtship glands, a cluster of smaller glands on the dorsal surface of the tail base (Noble 1929; Newman 1954; Sever 1985, 1989; Rupp and Sever 2017). These glands are situated where the female's head rests during the tail-straddling walk stage of courtship, and they secrete a variety of courtship pheromones, although the composition and effect of these pheromones are not as well studied as those from mental glands (Herrboldt et al. 2021). This caudal courtship gland is ubiquitous in male plethodontid salamanders,

occurring even in species inferred to have secondarily lost the mental gland (e.g., Houck and Sever 1994). In some species (e.g., some *Eurycea*), males display seasonally elongate cirri used in chemoreception and enlarged premaxillary teeth that pierce the upper lip and assist in courtship pheromone delivery from the mental gland (e.g., Noble 1929; Stewart 1958; Arnold 1977; Sever 1979). Finally, males of yet other plethodontid salamander species exhibit hypertrophied jaw musculature that appears to be related to mate guarding or other agonistic behaviors (Deitloff et al. 2014; Siegel et al. 2020; Wang et al. 2023). Together, these traits lead to gross morphological differences between the sexes in many, but not all, species of plethodontid salamanders.

For most species, the presence of the sexually dimorphic traits described above are invariant within a sex. However, in the Two-Lined Salamander (*Eurycea bislineata*) species complex, several evolutionary lineages exhibit morphologically distinct alternative reproductive tactics (ARTs) among males: "searching" males have a mental gland, elongate cirri, and protruding premaxillary teeth, but "guarding" males (originally described as "Morph A") lack these traits (Sever 1979), are larger (Bruce 1988; Pierson 2019), and have hypertrophied jaw musculature, resulting in relatively wider heads (Sever 1979; Pierson 2019; Pierson et al. 2019; Rainey et al. 2021). Sever (1989) found caudal courtship glands to be present in both male forms. These two distinct phenotypes reflect discrete differences in reproductive behavior: searching males primarily locate and court females in terrestrial environments, whereas guarding males exhibit mate-guarding behavior at or

near aquatic nesting sites in streams (Pierson 2019; Pierson et al. 2019). These morphologically distinct ARTs are unique among amphibians, but many important aspects of this system—including whether the ARTs are genetically or environmentally determined and whether they are plastic or fixed across the lifetime of an individual male—have not yet been described.

The *E. bislineata* species complex has a reticulate evolutionary history, and because the current taxonomy does not reflect true diversity, candidate species are sometimes referred to informally by mitochondrial lineage names (Jacobs 1987; Kozak et al. 2006; Pierson et al. 2023b). To date, ARTs have been described from four of these groups: Lineages E, J, M, and L (Sever 1979; Pierson et al. 2019, 2022). These four taxa are not monophyletic, and it remains unresolved whether the phylogenetic distribution of ARTs is the consequence of a shared ancestral polymorphism, convergent evolution, or introgressive hybridization. To evaluate these possibilities, it would be helpful to thoroughly catalog the various phenotypes associated with these ARTs. However, detailed descriptions of histological differences among ARTs are so far limited to a single species—populations from the southern Appalachian Mountains often referred to as Lineage M (Kozak et al. 2006; Pierson et al. 2019)—and no descriptions have been made of osteological differences from any of the polymorphic species. Thus, to better describe variation in head and skull morphology, we collected histological and osteological data from Lineages J and M (*Eurycea* cf. *wilderae*) and Lineage L (*Eurycea cirrigera*).

MATERIALS AND METHODS
Specimen Collection

We used three sources of specimens for our study. For all histological data, we used salamanders collected for previous behavioral and morphological research described in Pierson et al. (2019) and Rainey et al. (2021) and maintained at Lock Haven University (Pennsylvania, USA). For histological analyses of heads, we used specimens that included representatives of Lineage J collected from Paint Creek, Tennessee, USA ($n = 7$ searching males, 6 guarding males, and 2 females) and representatives of Lineage M collected from Davenport Gap, Tennessee ($n = 3$ searching males, 8 guarding males, and 1 female). For histological analyses of the caudal region, we used specimens that included representatives of Lineage J collected from Paint Creek ($n = 6$ searching males, 5 guarding males, and 6 females) and representatives of Lineage M collected from Davenport Gap ($n = 4$ searching males, 5 guarding males, and 7 females). We collected all salamanders during the courtship season (either October or January) and euthanized them shortly after the conclusion of laboratory trials (either November or February).

For osteological data collected by clearing and staining, we used new specimens collected by one of us (TWP). These included representatives of Lineage L collected from Athens, Georgia, USA, in November ($n = 1$ searching male and 1 guarding male) and representatives of Lineage M collected from the upper Tallulah River, Georgia, in December ($n = 1$ searching male and 1 guarding male). Finally, for osteological data collected by microcomputed tomography (microCT) scanning, we used specimens collected for an earlier ecological study (Hicks and Pearson 2003) and accessioned at the Georgia Museum of Natural History (GMNH).

These included two searching males (GMNH 43947 and GMNH 43944) and two guarding males (GMNH 43940 and GMNH 43945), all of which were collected in late September from a single plot in Pisgah National Forest, North Carolina, USA, and most likely represent Lineage J. All specimens in our study were fixed in 10% formalin and stored long term in 70% ethanol.

Histological Processing

For histological analyses of head tissues, we dissected tissues in two ways. For some specimens, we dissected half of the lower jaw and the surrounding tissue, whereas for others, we dissected the entire lower jaw. We decalcified all jaw tissues and embedded them in paraffin. We cut tissues into cross sections at 10 μm by using a rotary microtome (Leica Jung Biocut 2035) and stained the resulting cross sections by using standard histological procedures (Presnell and Shreibman 1997). For most tissues, we used a quad stain that includes a periodic acid–Schiff (PAS) reaction to identify carbohydrates, Alcian blue (pH = 2.0) to identify mucopolysaccharides, naphthol yellow to identify proteins, and Gill's hematoxylin for nuclear DNA (Floyd 1990; Staub and Paladin 1997). Typically, mental gland secretions react strongly positive for the PAS (PAS+) and negative for Alcian blue and nuclei stained with hematoxylin are observable along the periphery of the gland at the base of the secretory cells. We observed three regions from these cross sections: posterior to the mandibular symphysis (i.e., the submandibular region where the mental gland is typically found), anterior to the mandibular symphysis, and lateral to the mandible. Finally, we cut one tissue sagittally at 10 μm and stained it with hematoxylin and eosin. After staining, we observed all slides using a light microscope (Leica DMC) and captured images with a digital camera (Canon EOS Rebel 5).

For histological analyses of the caudal region, we dissected a 2–3 mm length of dorsal skin and muscle tissue above the cloacal region. We again cut these tissues into cross sections at 10 μm using a rotary microtome. We stained tissues with either hematoxylin and eosin for general cytology or with PAS reaction to identify carbohydrates and counterstained with Alcian blue (pH = 2.0) to identify mucopolysaccharides. Caudal courtship glands are eosinophilic similar to granular glands, but also react positively to PAS and negatively to Alcian blue (Sever 1985, 1989; Sever and Siegel 2015). They are also characterized by columnar cells with basal nuclei and secretory granules during the breeding season and with more cuboidal cells with central nuclei before the start of the breeding season (Sever 1989). We observed and photographed slides by using a light microscope (Leica DMi8) and captured images with a Leica DM2900 camera.

Cleared and Stained Specimens

We cleared and stained specimens following Dingerkus and Uhler (1977), with some modifications described in Crookston (2022). We skinned and eviscerated all specimens to remove any excess pigmentation that would stain soft tissues and obscure the elements of interest. Next, we moved specimens through a graded series of ethanol solutions of increasing concentration (75, 90, and 100%) for 12 h each and then we immersed specimens in a saturated solution of

Alcian blue, ethanol, and glacial acetic acid for 48 h. Following this step, we moved the specimens down through the same ethanol series and rinsed them with deionized water. We then transferred specimens to a 1% trypsin and sodium borate solution for 24 h, and we repeated this step two more times with fresh trypsin until the soft tissues were translucent and limp. We then immersed the specimens in a 100% Alizarin red and 0.5% potassium hydroxide (KOH) solution for 12 h. After this step, we moved the specimens into a solution of 3% hydrogen peroxide (H_2O_2) and 0.5% KOH for 24 h to remove any remaining pigmentation. We repeated this step with increasing amounts of H_2O_2 if necessary. We then moved specimens through a graded series of KOH and glycerol solutions (3:1, 1:1, and 1:3) before storing them in 100% glycerol with grains of thymol as a preservative. After clearing and staining, we observed specimens by using a dissecting microscope (Olympus SZX7) and captured images with an attached digital camera (Olympus EP50).

microCT Scanning

We collected high-resolution microCT data of four specimens using a GE v|tome|x M 240 system at the Nanoscale Research Facility (University of Florida, Gainesville, USA). We conducted scans using a 240 kv X-ray tube containing a diamond-tungsten target, with the voltage between 60 and 80 kV and the current between 130 and 180 μA, adjusted for each scan to maximize absorption range for each specimen. We processed raw X-ray data by using GE's proprietary datos|x v2.3 software, producing tomogram and volume files with final voxel resolutions of 12 μm. We imported these microCT volume files into VG StudioMax v3.5 (Volume Graphics, Heidelberg, Germany) and isolated the skull and individual cranial elements by using the suite of segmentation tools in VG StudioMax. We recorded linear measurements for skull length, tooth height, and tooth width by using the polyline length tool in VG StudioMax (Supplemental Fig. S1, available online). We measured skull length from the occipital condyle to the tip of the snout (anterior extent of the premaxilla). Because tooth size gradually decreases from the anterior-to-posterior extent of the maxilla and dentary, we measured every functional tooth (i.e., ankylosed to bone) to calculate average and maximum tooth height and width. Because nearly all premaxillary teeth on both searching males were undergoing replacement and no longer attached to bone, we measured only the longest tooth of each specimen, regardless of attachment status. Because the teeth on the vomer and isolated paravomerine tooth patch were similar in size, we randomly selected and measured six teeth (i.e., three from each element) to calculate average vomerine/paravomerine tooth length. We deposited image stacks (TIFF) and high-fidelity mesh files (.stl) of the skull for each specimen in MorphoSource (DOIs: http://dx.doi.org/10.17602/M2/M607322; http://dx.doi.org/10.17602/M2/M607331; http://dx.doi.org/10.17602/M2/M607347; http://dx.doi.org/10.17602/M2/M607349).

Results

Histology of Glands

In general, we identified and refer to four types of glands: (1) mucous glands; (2) granular glands; (3) mental glands;

and (4) caudal courtship glands. Mucous glands typically stain positively with Alcian blue and are thus blue in color. However, we observed putative mucous glands that were similar in shape and location to those described from other *Eurycea* (Sever 1975, 1979), but variable in both the intensity of this Alcian blue stain and in partial reactivity to the PAS treatment—including some from within the same cross sections as typical mucous glands. Some of these glands had a homogeneous staining pattern in which the gland stained positive for both Alcian blue and PAS in the same region, causing it to appear purple; in other glands, the distribution of these positive stains was heterogeneous across the glands, with some secretory packets Alcian blue positive and others PAS+. To be conservative and to remain agnostic about their function, we refer to all of these putative mucous glands with somewhat atypical staining patterns simply as modified mucous glands. Typical granular glands are naphthol yellow positive and sometimes weakly PAS+, causing them to appear yellow or light pink. We also encountered some granular glands that were more strongly PAS+, and we follow the precedent of previous studies (Staub and Paladin 1997; Sever et al. 2016a) in referring to these as modified granular glands. Mental glands and caudal courtship glands react strongly with the PAS treatment (PAS+), but are negative for Alcian blue and slightly positive for naphthol yellow (Staub and Paladin 1997); this causes them to appear bright pink or magenta in color. Below, we describe the distribution of each of these gland types among tissues in females, guarding males, and searching males. The distribution of these glands was consistent between Lineage J and Lineage M.

Submandibular region.—In females, this ventral submandibular region (i.e., posterior to the mandibular symphysis) mostly contained mucous glands and some granular glands (Fig. 1A–C). We noted differences in the reactivity to Alcian blue and PAS, but mostly within the same cross section (e.g., compare Fig. 1B to Fig. 1C). Guarding males likewise had a similar mixture of mucous glands and granular glands (Fig. 1D–F), with these alternating in some specimens (Fig. 1F). By contrast, searching males showed traditional mental glands (Fig. 1G–I) that were much larger, appeared to have much more secretory material, and had more obvious nuclei than any glands found in females and guarding males (see also Supplemental Fig. S2, available online). The portion of the mental gland that extended posteriorly drained to the anterior part of the gland; the mental gland secreted through ducts in the epidermis, with some extending under the mandible to pores exiting at the base of the chin, consistent with the description of the mental gland in Sever (1979).

Anterior to mandibular symphysis.—In females, this anterior region primarily contained typical mucous glands and modified mucous glands (Fig. 2A–C). In general, the mucous glands were located on the ventral surface and the modified mucous glands were layered above them more dorsally. Sections from guarding males appeared very similar to those from females, with both mucous glands and modified mucous glands (Fig. 2D–F). The same was true for searching males, except that some specimens had several larger glands that were more PAS+, likely indicating the anterior most extent of the mental gland (Fig. 2G,I).
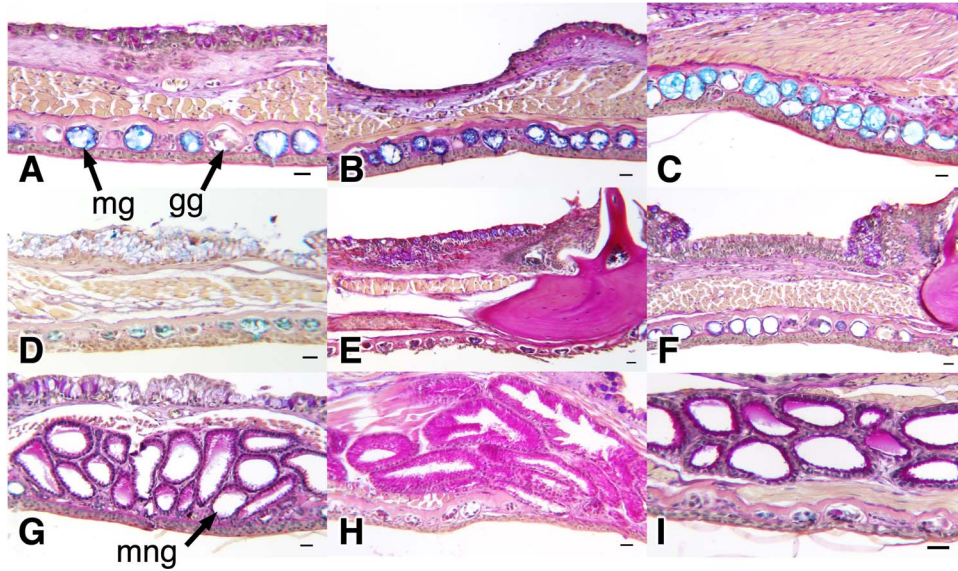
Fig. 1.—Representative photomicrographs of cross sections of the area posterior to the mandibular symphysis, the submandibular region in which the mental gland is found, in *Eurycea* cf. *wilderae*. The ventral surface is on the bottom of each image. (A–C) Females. (D–F) Guarding males. (G–I) Searching males. All staining was done using the quad stain. mg = mucous gland; gg = granular gland; mng = mental gland. Scale bars, 100 μm.

Lateral to mandible.—In females, guarding males, and searching males, the region lateral to the mandible consisted primarily of mucous glands and modified mucous glands, with the latter primarily clustered toward the dorsal edge (Fig. 3). In most females and guarding males, a few modified mucous glands were clustered near the dorsal edge followed by typical mucous glands distributed ventrally (e.g., Fig. 3A,C), although variation was common (Fig. 3F). In searching males, these modified mucous glands sometimes extended further ventrally (Fig. 3G). Similar to the other regions described above, there was also considerable variation in reaction intensity to both the Alcian blue and PAS stains among sections.

Caudal region.—In the caudal region, we identified mucous glands, granular glands, modified granular glands, and caudal courtship glands (Fig. 4). In all females ($n = 13$), we found mucous glands and granular glands only (Fig. 4A, D); these glands were also present in males. In some females,

we noted modified granular glands (Fig. 4D). We found caudal courtship glands in all guarding males euthanized in November ($n = 6/6$; Fig. 4B), but no guarding males euthanized in February ($n = 0/4$). Similarly, we found caudal courtship glands in most searching males euthanized in November ($n = 3/5$; Fig. 4C) and no searching males euthanized in February ($n = 0/5$).

Cleared and Stained Specimens

In the lateral view of Lineage L, the guarding male had much more pronounced otic and squamosal crests on the skull than the searching male (Fig. 5A,B). It also appeared to have larger teeth on the premaxilla, maxilla, and dentary. In both the dorsal and ventral view, the skulls appeared superficially similar, although the nasal capsule of the searching male was slightly larger (Fig. 5C,D). In the ventral and lateral
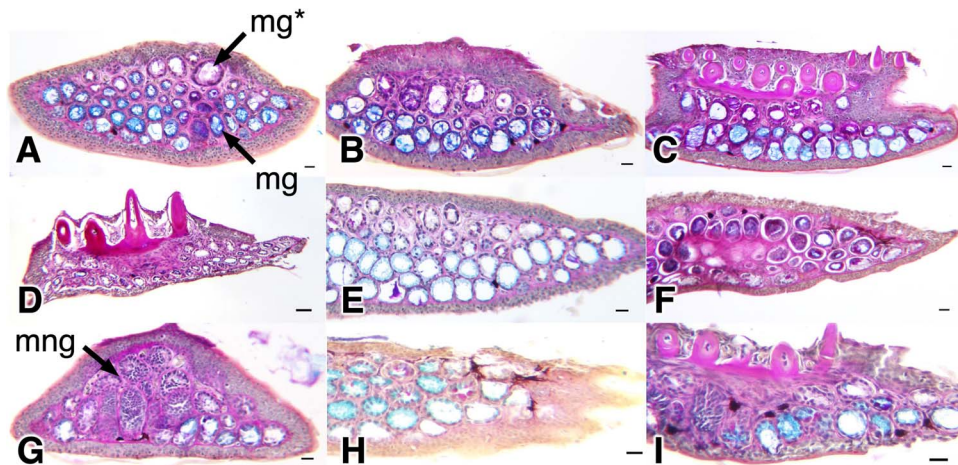

Fig. 2.—Representative photomicrographs of cross sections of the region anterior to the mandibular symphysis in *Eurycea* cf. *wilderae*. The ventral surface is on the bottom of each image. (A–C) Females. (D–F) Guarding males. (G–I) Searching males. All staining was done using the quad stain. mg = mucous gland; mg* = modified mucous gland; mng = mental gland. Scale bars, 100 μm.
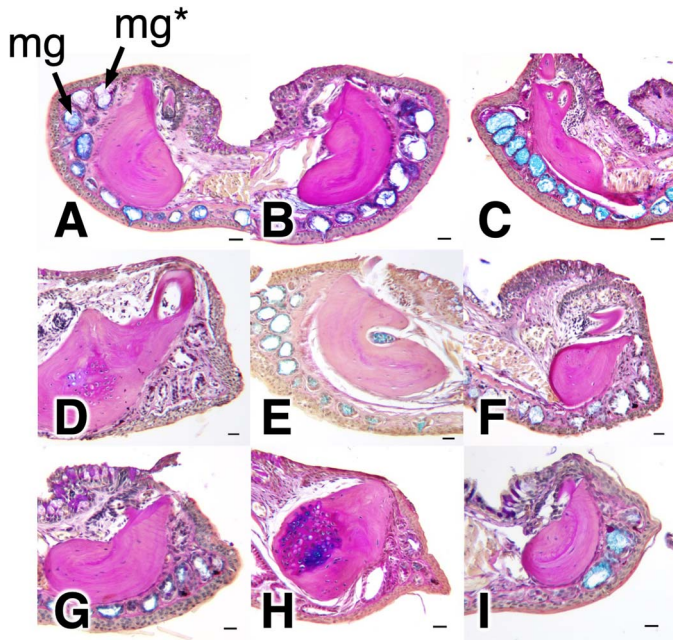
FIG. 3.—Representative photomicrographs of cross sections of the area lateral to the mandible in *Eurycea* cf. *wilderae*. The ventral surface is on the bottom of each image. (A–C) Females (D–F) Guarding males. (G–I) Searching males. All staining was done using the quad stain. mg = mucous gland; mg* = modified mucous gland. Scale bars, 100 μm.

In the lateral view of Lineage M, the guarding male again had much more pronounced otic and squamosal crests than the searching male (Fig. 6A,B). The guarding male also had larger teeth on the premaxilla, maxilla, and dentary. In the dorsal view, the prominent squamosal crests were visible on the guarding male, but not the searching male (Fig. 6C,D). In both the dorsal and ventral views, the dentary appeared more robust in the guarding male and the nasal capsule larger in the searching male (Fig. 6E,F). Similar to Lineage L, the posterior process of the maxilla and the anterior extent of the dentary were more gracile in the searching male. The posterior process of the maxilla tapered without dorsal curvature in the searching male, but it was highly curved in the guarding male. The coronoid process of the prearticular was also taller and more angular in the mandible of the guarding male compared to that of the searching male (Fig. 6A,B).

microCT Scanning

The microCT scans of skulls from Lineage J showed results qualitatively similar to those described from cleared and stained specimens of Lineages L and M described above (Figs. 7 and 8). Overall, the individual cranial elements of the guarding males were robust and more heavily ossified than those from searching males. The articulations between elements were also more extensive in the guarding males, including between the paired parietals, the paired frontals, and the bony elements forming the snout (articulations between the frontal, prefrontal, nasal, maxilla, and premaxilla). Otic and squamosal crests were prominent (but variable in size, being substantially larger in GMNH 43940) in guarding males (Fig. 7A,C) and absent in searching males (Fig. 7B,D). The maxillary and

views, the maxilla and dentary of the guarding male appeared more robust, whereas the posterior process of the maxilla and the anterior extent of the dentary (surrounding the mandibular symphysis) of the searching male were slender and gracile (Fig. 5A,B, E,F).
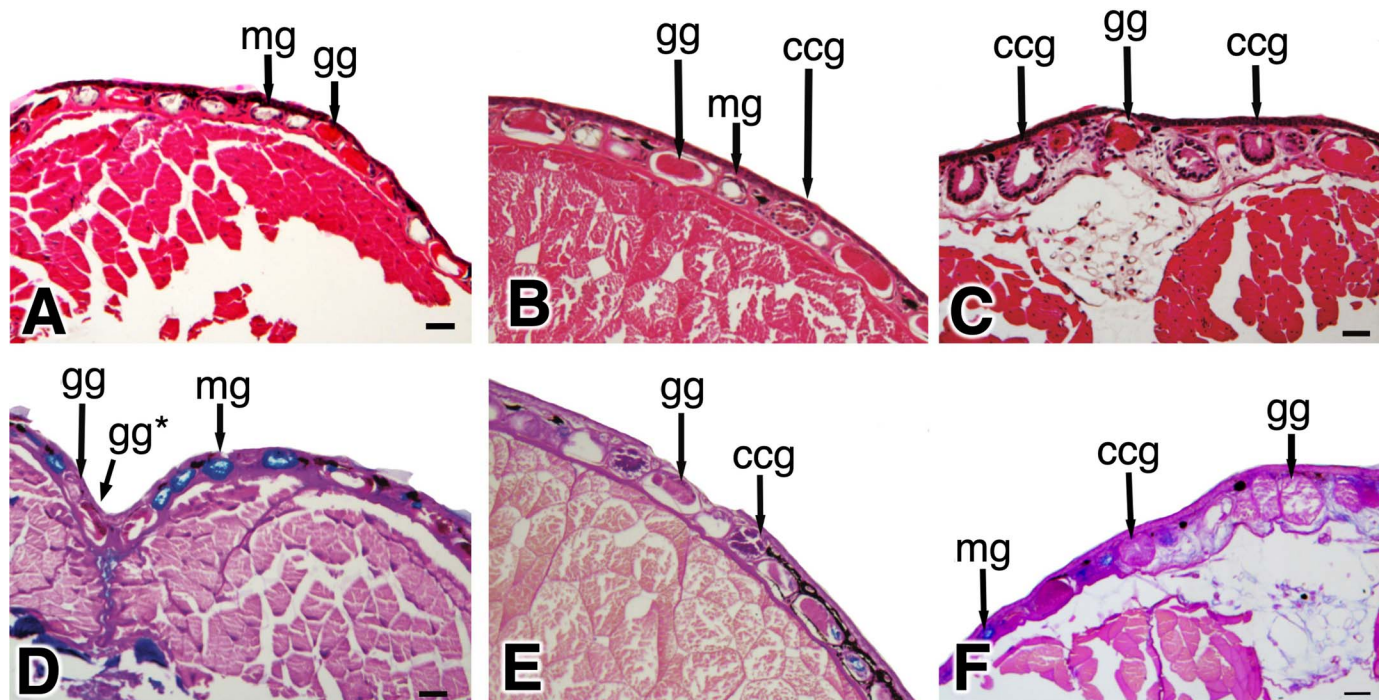

FIG. 4.—Representative photomicrographs of cross sections of the tail region dorsal to the cloaca in *Eurycea* cf. *wilderae*. The ventral surface is on the bottom of each image. (A–C) Representative images of tissues stained with hematoxylin and eosin from (A) a female, (B) a guarding male, and (C) a searching male. (D–F) Representative images of tissues stained with PAS and Alcian blue from (D) a female, (E) a guarding male, and (F) a searching male. mg = mucous gland; gg = granular gland; gg* = modified granular gland; ccg = caudal courtship gland. Scale bars, 50 μm.
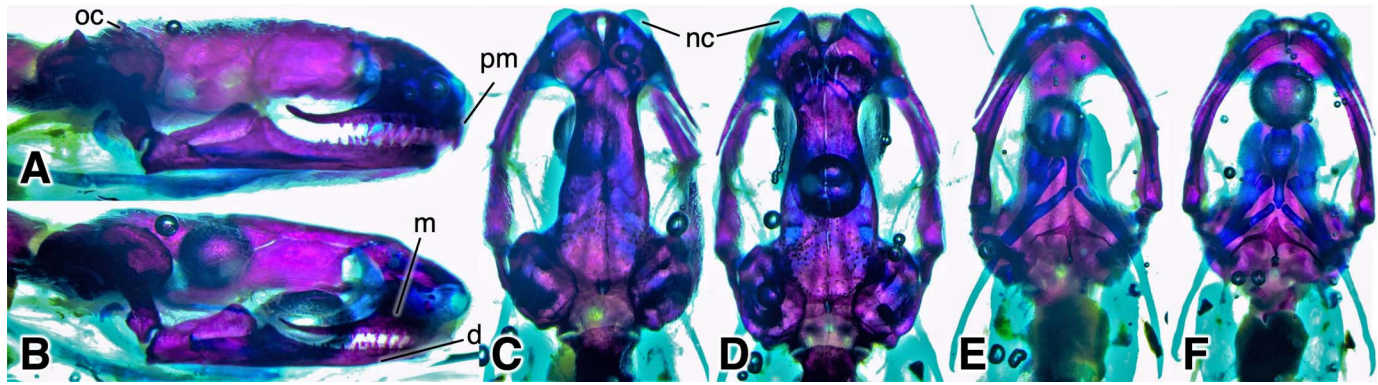
Fig. 5.—Photographs of cleared and stained skulls from *Eurycea cirrigera*, showing lateral (A–B), dorsal (C–D), and ventral (E–F) views. The images depict a guarding male (A, C, E) and a searching male (B, D, F) collected from Athens, Georgia. Note that small and large globular masses are bubbles and not part of the skull osteology. d = dentary; m = maxilla; nc = nasal capsule; oc = otic crest; pm = premaxilla.

dentary teeth were larger and more robust in guarding males than in searching males (Fig. 9; Table 1), despite having similar skull lengths (guarding males = 6.48 mm [GMNH 43945], 6.77 mm [GMNH 43940]; searching males = 6.84 mm [GMNH 43944], 6.50 mm [GMNH 43947]). By contrast, the premaxillary teeth were longer in the searching males (Table 1). Through visual examination under a dissecting scope, we confirmed that these premaxillary teeth are piercing the upper lip in the searching males, but not the guarding males. The vomerine and paravomerine teeth did not differ in size between guarding and searching males (Table 1). The canal for the passage of the nasolacrimal duct that forms at the junction of the maxilla, prefrontal, and nasal was larger in searching males (0.22 mm in GMNH 43944 and 0.27 mm in GMNH 43947) than in guarding males (0.13 mm in GMNH 43945 and 0.09 mm in GMNH 43940).

DISCUSSION

Together, our results help to better describe inter- and intrasexual soft-tissue differences in morphology and courtship glands in polymorphic populations of the *E. bislineata* species complex. Perhaps most notable, our results also provide the first evidence of substantial osteological differences between these male tactics. Among amphibians, the ARTs found in these *Eurycea* are unique in that they are accompanied by marked morphological differences, and our study provides new evidence that these traits extend to skull osteology. Below, we discuss each of our results in greater detail and describe their implications for understanding the ecology and evolution of ARTs in *Eurycea*.

Our examination of soft-tissue differences confirmed results from previous studies. In both Lineage J and Lineage M, we found traditional mental glands present only in searching males. This is consistent with the original description of this morphological polymorphism by Sever (1979), although that study described a single guarding male with a markedly reduced mental gland cluster entirely encased in the dermis. We encourage future studies to more closely examine the prevalence and structure of these reduced mental glands in some guarding males; such studies may provide insight into their physiology and development and reveal a role in chemical communication during courtship.

As previously described in *Eurycea* (Sever 1975, 1979), we found mucous glands to be abundant in the lower jaw of both sexes and both male reproductive tactics. However, we noted substantial variation in the strength of both PAS and Alcian blue staining in these glands—sometimes within the same cross section. There are a few possible explanations for these modified mucous glands. They could represent variation in staining intensity introduced during the preparation of the tissues (e.g., due to pH; Siegel et al. 2017). This hypothesis is partially supported by the apparent variation in stain intensity in tissues of known origin (e.g., bone, mental glands) in the same tissues (e.g., Fig. 1G vs. Fig 1H).
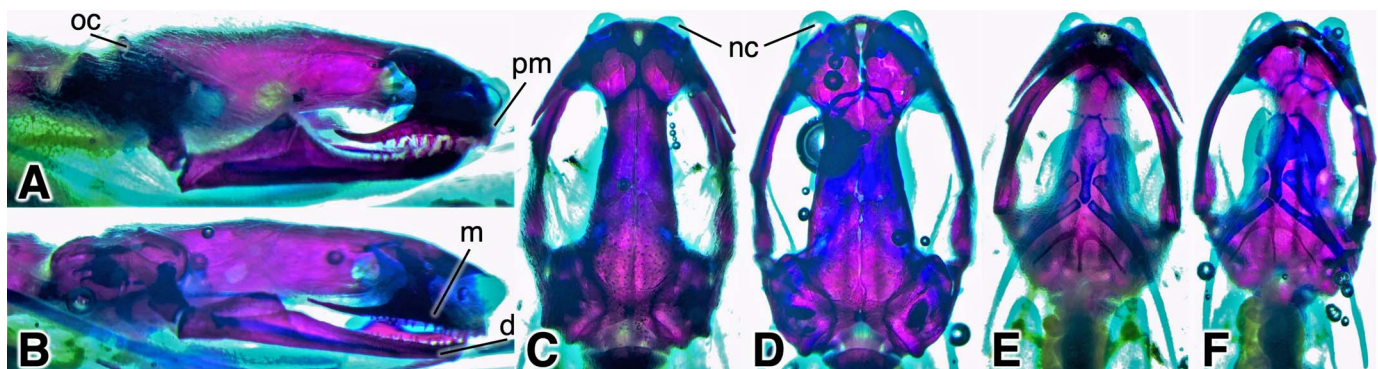

Fig. 6.—Photographs of cleared and stained skulls from *Eurycea* cf. *wilderae* showing lateral (A–B), dorsal (C–D), and ventral (E–F) views. The images depict a guarding male (A, C, E) and a searching male (B, D, F) collected from the Upper Tallulah River, Georgia. Note that small and large globular masses are bubbles and not part of the skull osteology. d = dentary; m = maxilla; nc = nasal capsule; oc = otic crest; pm = premaxilla.
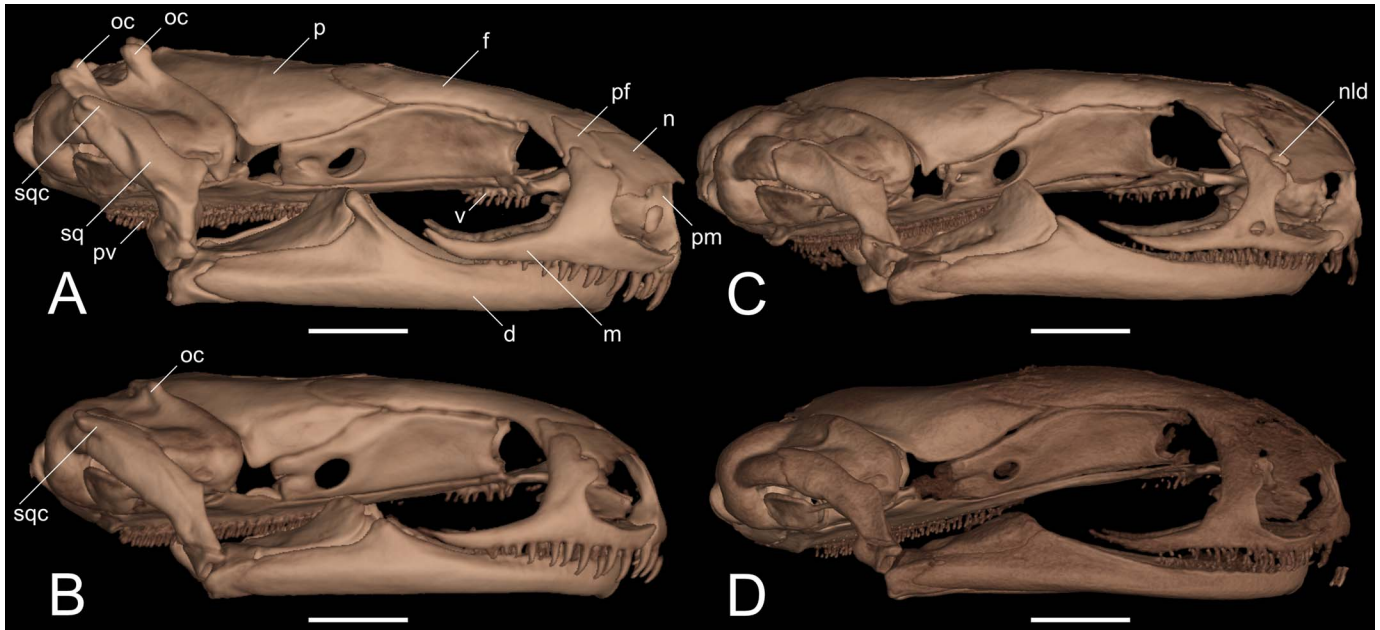
FIG. 7.—Skulls from *Eurycea* cf. *wilderae* collected from the Pisgah National Forest, North Carolina, and visualized via microCT scanning in lateral view. (A) GMNH 43940, guarding male; (B) GMNH 43945, guarding male; (C) GMNH 43944, searching male; and (D) GMNH 43947, searching male. d = dentary; f = frontal; m = maxilla; n = nasal; nld = nasolacrimal duct; oc = otic crest; p = parietal; pf = prefrontal; pm = premaxilla; pv = paravomerine teeth; sq = squamosal; sqc = squamosal crest; v = vomer. Scale bar, 1 mm.

Alternatively, these modified mucous glands could represent true variation in gland structure and secretory product not previously recognized. Variation in mucous glands is well known in frogs. For example, the rhacophorid *Polypedates maculatus* has two types of mucous glands that differ in secretory product and thus have different staining characteristics (Lillywhite et al. 1997), and mucous glands of the pyxicephalid *Amietia fuscigula* reacted variably to Alcian blue and PAS (Els and Henneberg 1990). This variation, found both between glands and within individual glands, was interpreted to be a consequence of differences in gland developmental stages (Els and Hennberg 1990). Several ranids have a second type of specialized mucous gland, found only in males, that is hypothesized to have a signaling function (Brizzi et al. 2002).

Although variation in mucous glands is not as well studied in salamanders, several examples do exist. The salamandrid *Triturus karelinii* has two types of mucous glands—both of which have a PAS+ reaction that is variable between and within glands (Bingol-Ozakpinar and Murathanoglu 2011). Furthermore, these glands have granular as well as nongranular secretory products (Bingol-Ozakpinar and Murathanoglu 2011). In the plethodontid *Ensatina eschscholtzii*, a seemingly unusual mucous gland was described as a mucus-producing granular gland (Fontana et al. 2006). Our results on variable mucous glands in *Eurycea* suggest that mucous glands may be a more heterogeneous class of glands than previously noted. An in-depth study of mucous gland variation across amphibians would be a major contribution to the field, because it would provide a more robust foundation upon which to standardize descriptions among taxa.

We found typical caudal courtship glands present only in searching and guarding males. One surprising result is that we observed these glands only in the specimens that we collected in October and euthanized in November and not in any of the animals we collected in January and euthanized in February. Sever (1989) does describe seasonal changes in these glands, with their size larger and secretory products greater in the breeding season than out of it. However, he found obvious caudal courtship glands in both searching and guarding males from Lineage M collected in March, and this is certainly still within the courtship period for guarding males (Reagan 1984; Pierson et al. 2023a). We suspect that these caudal courtship glands were not truly absent in our samples, but rather that they were reduced and harder to differentiate from other glands—perhaps as an artifact of the maintenance of these salamanders in laboratory conditions. Because searching and guarding males may differ in the average timing of courtship (Reagan 1984; Pierson 2019), we encourage future work to more closely examine the timing of seasonal changes in both mental and caudal courtship glands.

In some females, we also noted what appeared to be granular glands that were more PAS+ than is typical. Similar glands have been described in other male and female plethodontid salamanders as "modified granular glands" (Staub and Paladin 1997; Sever et al. 2016b) and "caudal courtship-like glands" (Rollins and Staub 2017). Thus, our work raises the possibility of a greater diversity of scattered pheromone-producing glands than is currently recognized. We hypothesize that these glands could be important for female–male communication during courtship (e.g., during head-sliding and -pulling behaviors, male *Eurycea* have their nasolabial grooves in contact with the female; Kozak 2003), which may be more common than traditionally thought (Staub et al. 2020). Broader surveys of salamanders could help place these hypotheses in a phylogenetic context, and molecular work to analyze the content of these glands would be critical to understanding their function (e.g., Fontana et al. 2007; Herrboldt et al. 2021; DeBruin et al. 2023).
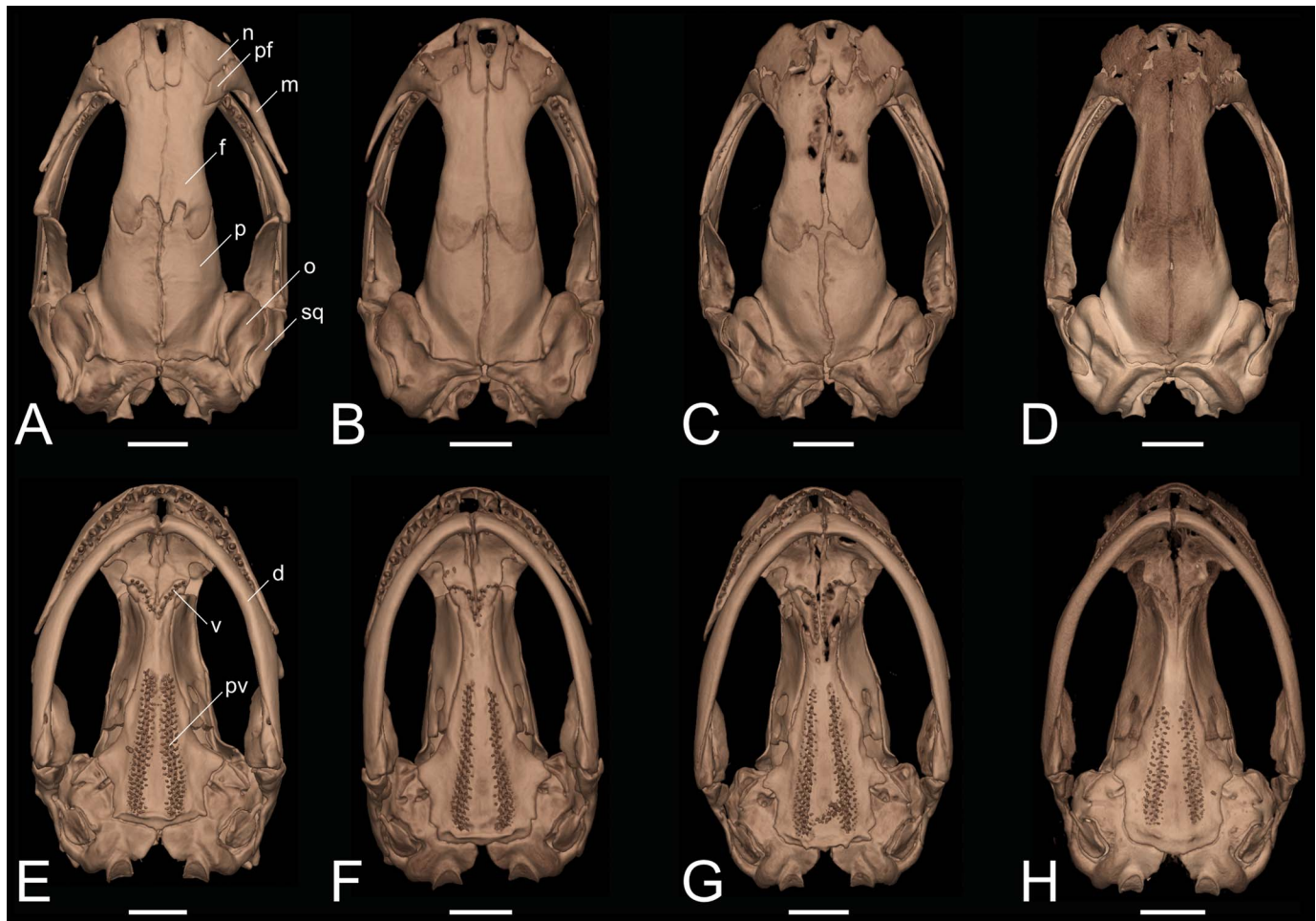
Fig. 8.—Skulls from *Eurycea* cf. *wilderae* collected from the Pisgah National Forest, North Carolina, and visualized via microCT scanning in dorsal (A–D) and ventral (E–H) views. (A, E) GMNH 43940, guarding male; (B, F) GMNH 43945, guarding male; (C, G) GMNH 43944, searching male; and (D, H) GMNH 43947, searching male. d = dentary; f = frontal; m = maxilla; n = nasal; o = otic capsule; p = parietal; pf = prefrontal; pv = paravomerine teeth; sq = squamosal; v = vomer. Scale bar, 1 mm.

We identified substantial differences in the cranial anatomy between guarding and searching males of Lineages J, M, and L. The skulls of guarding males were more heavily ossified, possessed pronounced otic and squamosal crests, and had larger maxillary and dentary teeth. Searching males had longer premaxillary teeth and a larger canal for the passage of the nasolacrimal duct. The prominence of otic and squamosal crests in guarding males most likely reflects the muscle attachment sites for their hypertrophied jaw musculature (Sever 1979; Siegel et al. 2020). The *M. levator mandibulae externus* (LME) is one of the cranial muscles responsible for jaw closure, and it is hypertrophied in guarding males (Sever 1979; Siegel et al. 2020). It originates on the posterior otic capsule (exoccipital) and posterior flange of the squamosal, and it inserts on the prearticular of the mandible (Lauder and Shaffer 1985; Deban and Wake 2000). We hypothesize that in guarding males, the prominent dual crests of the otic capsule that continue onto the squamosal act as additional surface area for muscle attachment. These projections anchor the hypertrophied LME and allow for dorsolateral expansion of the muscle body, which improves cross-sectional area, thereby increasing bite force (Deban and Richardson 2017). These same skull features were described by Wake (1966) as

"[d]ouble [otic] crests" in *Eurycea aquatica* and *E. bislineata*—two species in which all males have hypertrophied jaw musculature (Graham et al. 2013; Siegel et al. 2020). Wake (1963, 1966) also recognized sizeable otic crests in *Aneides*, which exhibit jaw muscle hypertrophy associated with agonistic behavior (Staub 1993, 2021), and in *Pseudotriton*, for which there is preliminary evidence of agonistic behavior in males and sexual dimorphism in head shape and head size (Wang et al. 2023). A comparative analysis of skull shape and cranial muscle variation among plethodontid salamanders and between sexes—especially with the high-resolution data now enabled by the advent of contrast-enhanced soft-tissue microCT scanning (Gignac et al. 2016)—may help further explain the evolutionary history of these structures and their relationship to reproductive behaviors.

The enlarged maxillary and dentary teeth in guarding males may also be associated with agonistic behavior. Noble (1927) identified elongated maxillary and dentary teeth as a secondary sexual character in males of some *Desmognathus*, *Eurycea*, and *Hydromantes*, and Stewart (1958) confirmed that the maxillary and dentary teeth in male *E. bislineata* were larger and less numerous than those in females. In addition to cranial crests, hypertrophied jaw muscles,
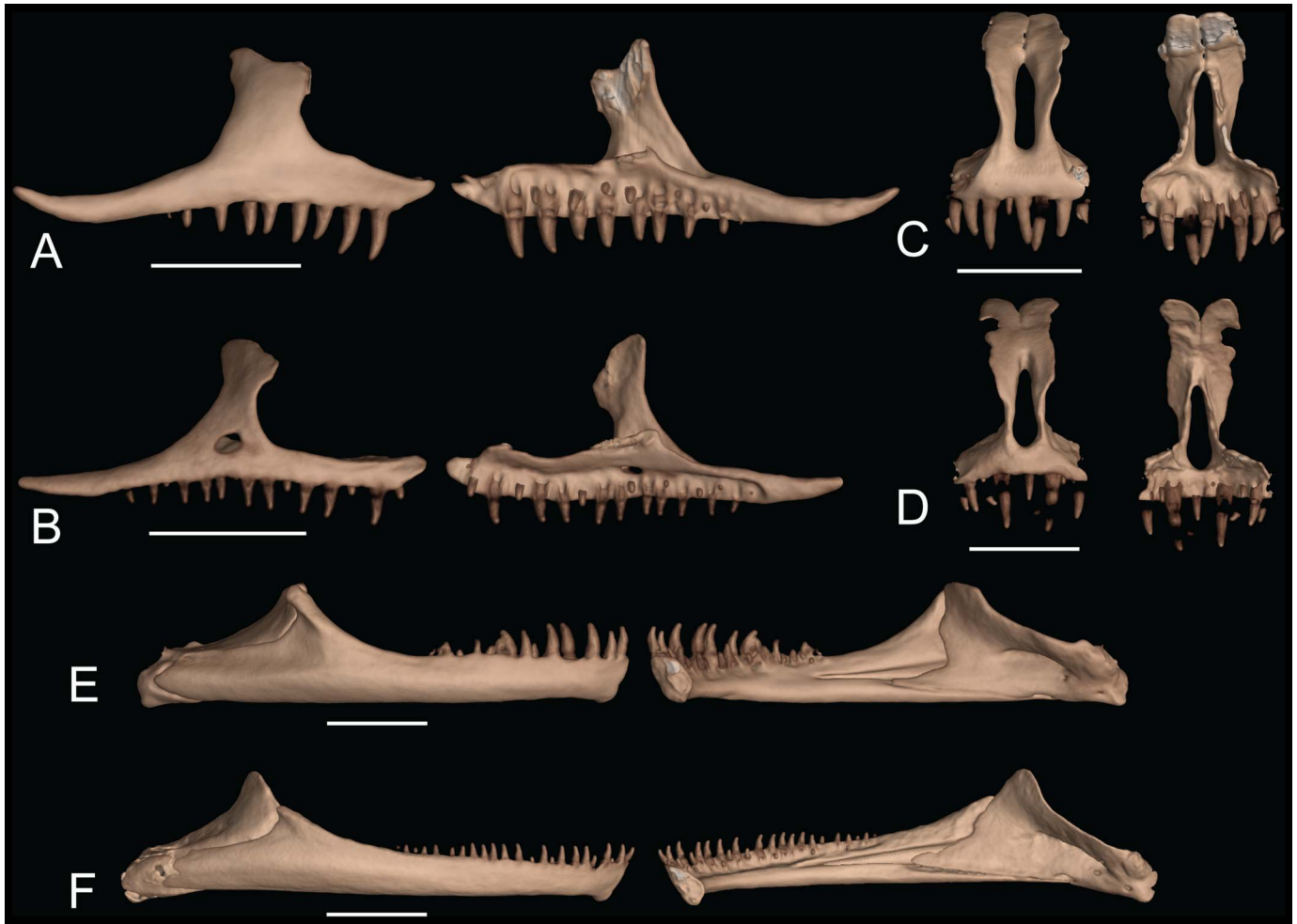
FIG. 9.—Comparison of selected cranial bones from *Eurycea* cf. *wilderae* collected from the Pisgah National Forest, North Carolina, and visualized via microCT scanning in lateral (left) and lingual (right) views. (A) Right maxilla of a guarding male (GMNH 43940) in lateral and lingual view. (B) Right maxilla of a searching male (GMNH 43944). (C) Paired premaxillae of the same guarding male. (D) Paired premaxillae of the same searching male. (E) Right lower jaw of the same guarding male. (F) Right lower jaw of the same searching male. Scale bar, 1 mm.

and agonistic behavior, enlarged teeth have been reported on the maxilla and dentary in the males of several *Aneides* species (Wake 1963). Similarly, Clemen and Greven (2009) reported sexually dimorphic dentition in the hynobiid *Pachyhynobius shangchengensis*, with males possessing upper and lower jaw teeth that are large and chisel-like, in contrast to females that have a typical dentition. Males of *P. shangchengensis* are larger and more robust than females, have displayed aggressive behavior in captivity, and may engage in intrasexual combat (Xiong et al. 2019).

We observed longer premaxillary teeth in searching males than in guarding males. Elongated, monocuspid premaxillary teeth have been identified in the males of many plethodontid salamanders (Noble 1929; Noble and Pope 1929; Wake 1966; Ehmcke and Clemen 2000b; Greven et al. 2004), including in the *E. bislineata* species complex (Noble 1929; Sever 1979). These elongated premaxillary teeth are used by males during courtship to abrade the female's skin before the transfer of pheromones secreted by the mental gland (Sever 1979; Houck and Arnold 2003). Stewart (1958) demonstrated that the sexually dimorphic premaxillary teeth of *E. bislineata* are seasonally induced, with males possessing elongated, monocuspid teeth only during the breeding season. Changes in premaxilla dental morphology of male

TABLE 1.—Tooth size variation between guarding and searching males of Lineage J of the *Eurycea bislineata* species complex. TH = tooth height; TW = tooth width. The average and maximum tooth size (in mm) is reported for all specimens (avg/maximum).

Tactic	Specimen	Maxilla TH	Maxilla TW	Dentary TH	Dentary TW	Premaxillary TH	Vomerine TH
Guarding	GMNH 43945	0.24/0.36	0.12/0.14	0.24/0.39	0.13/0.17	0.36/0.41	0.10/0.14
	GMNH 43940	0.25/0.35	0.12/0.16	0.28/0.39	0.14/0.18	0.40/0.44	0.11/0.15
Searching	GMNH 43944	0.16/0.23	0.08/0.11	0.16/0.19	0.07/0.10	—[a]/0.53	0.10/0.14
	GMNH 43947	0.17/0.21	0.08/0.09	0.18/0.20	0.07/0.11	—/0.54	0.11/0.14

[a] Dashes indicate that no data were collected. See Methods.

plethodontids are likely induced by elevated androgen levels during the breeding season in temperate species (Noble and Pope 1929; Stewart 1958). Tropical plethodontid species lack distinct mating seasons and possess dimorphic premaxillary teeth at all times; these are likely induced and maintained by localized androgen receptor expression only within the premaxillary dental lamina, which proliferates into tooth buds (Ehmcke and Clemen 2000a; Ehmcke et al. 2003). The divergent tooth sizes between guarding and searching males indicate considerable modularity in the dental development program between the units of the mouth (premaxilla, maxilla and dentary, and palate), warranting further investigation within this species complex in the context of seasonality, hormonal regulation, and function.

The canal for the passage of the nasolacrimal duct is roughly twice as large in searching males as in guarding males, which may be a by-product of the reduced ossification of the skull in searching males. The nasolacrimal duct acts as a passage route for secretions from the orbital gland into the nasal and vomeronasal organ cavities and is hypothesized to enhance vomeronasal organ function (Hillenius 2000). Therefore, the enlarged passage in searching males may have implications for enhanced pheromonal communication, but the function of the orbital glands and nasolacrimal duct in plethodontid salamanders requires further study (Siegel et al. 2018), especially within *Eurycea* (Noble 1929; Siegel et al. 2017).

In other organisms, ARTs can be either fixed or plastic across the lifetime of an individual (Gross 1996; Oliveira et al. 2008). In amphibians, nearly all examples of ARTs are plastic, with some males able to switch between tactics on very short time scales in response to differences in social context or body condition (Zamudio and Chan 2008). The prominent, discrete differences in reproductive morphology between searching and guarding males in the *E. bislineata* species complex likely exclude the possibility of plasticity within a breeding season. However, many of these secondary sexual characteristics (e.g., cirri, mental glands, jaw musculature, premaxillary teeth) hypertrophy and atrophy seasonally (Sever 1979). Because guarding males are on average slightly larger than searching males, Bruce (1988) hypothesized that the two phenotypes could result from an ontogenetic shift, with younger searching males returning as older guarding males in later breeding seasons. Although not conclusive, we believe that our demonstration of markedly different skull structure between searching and guarding males makes this unlikely. However, because seasonal changes in skull morphology have been reported in other vertebrates (i.e., Dehnel's phenomenon; Lázaro et al. 2017), we cannot exclude this possibility and encourage future researchers to test our hypothesis by examining *Eurycea* skulls collected across seasons.

Supplemental Material

Supplemental material associated with this article can be found online at https://doi.org/10.1655/Herpetologica-D-23-00046.S1.

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