FLOWER AND FRUIT DEVELOPMENT OF THREE SPECIES OF HYDROPHYLLACEAE SHEDS NEW LIGHT ON FLOWER EVOLUTION IN HYDROPHYLLACEAE

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Premise of research. Hydrophyllaceae are characterized by simple floral organization contrasting with a peculiar floral architecture resulting from complex compartments formed by stamen–corolla tube modifications. Additionally, the internal ovary architecture of Hydrophyllaceae shows significant variation, while the developmental trajectory of the gynoecium is relatively conserved. Despite insights from recent studies of the flower and fruit evolution of the family, there are only a few ontogenetic studies of Hydrophyllaceae, and a complete understanding of the underlying processes has not yet been achieved.

Methodology. Here, we use scanning electron microscopy and micro–computed tomography to investigate the flower and fruit ontogeny of two genera of Hydrophyllaceae, Emmenanthe and Pholistoma, with a particular focus on the gynoecium and modifications of the stamen–corolla tube.

Pivotal results. Our results complement two previously published data sets, broadening our understanding of Hydrophyllaceae evolution. Hydrophyllaceae comprise only a few monotypic or small genera, but their floral evolution appears to be remarkably complex, in terms of both gynoecial structure and perianth modifications. We find 10 stamen–corolla tube modifications, although these may be rudimentary in species previously considered as lacking them altogether (e.g., Emmenanthe). The relative conservation of perianth architecture contrasts with the highly variable internal ovary architecture of Hydrophyllaceae. There is considerable divergence in ovule/seed number, as well as in the details of (parietal) placentation and septation, and we propose a hypothetical evolutionary series for the internal ovary architectural diversity of Hydrophyllaceae.

Conclusions. We propose that—starting from a fairly conserved floral organization—minor heterochronous shifts in both perianth and ovary development can explain most of the morphological diversity found in the flowers and fruits of Hydrophyllaceae.

Keywords: Boraginales, gynoecium, heterochrony, internal ovary architecture, placentation, stamen–corolla tube modifications.

Introduction

Boraginales are divided into two major clades: Boraginales I and II (Weigend et al. 2014). Boraginales I comprise Codonaceae, Wellstediaceae, and Boraginaceae, the most species-rich family of the entire order. Boraginales II comprise eight families: Hydrophyllaceae, Namaceae, Ehretiaceae, Lennoaceae, Heliotropiaceae, Cordiaceae, Coldeniaceae, and Hoplestigmataceae (Luebert et al. 2016). Fruit morphology has traditionally been considered an important character complex for their familial classification. In both major clades, the early-diverging lineages (Codonaceae/Wellstediaceae and Hydrophyllaceae/Namaceae, respectively) bear capsular fruits, while the remaining lineages, with the sole exception of Lennoaceae (Luebert et al. 2016), have either mericarps (i.e., dispersal units of schizocarpic or eremocarpic fruits [nutlets] formed by part of a carpel; Hilger 2014; Jeiter et al. 2018) or drupes. Details of fruit morphology, development, and evolution have been the focus of several recent studies of Boraginales (Gottschling 2004; Gottschling et al. 2014; Luebert et al. 2016; Jeiter et al. 2018; Heigl et al. 2020; Vasile et al. 2021). Indehiscent fruits have been the primary focus of these studies, while capsular fruits have largely been neglected until recently (Jeiter et al. 2016; Vasile et al. 2021). The phylogenetic results showing the independent evolutionary trends from

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capsules to nutlets in both major clades (Weigend et al. 2014) have raised questions about the mechanisms for the reduction and subsequent stabilization of low seed numbers in capsular fruits. Hydrophyllaceae, as the largest capsule-bearing family within Boraginales, was identified as an ideal object for studying such evolutionary trends.

Hydrophyllaceae comprise some 240–260 species in 12 genera (Luebert et al. 2016) distributed in North and South America (Vasile et al. 2020). The family is divided into three clades. The largest and morphologically most diverse clade is the Romanzoffiinae, with the genera Phacelia Juss. and Romanzoffia Cham. The second-largest clade is the Hydrophyllinae, comprising the genera Hydrophyllum L., Nemophila Nutt. ex W.P.C.Barton, Pholistoma L., Eucrypta Nutt., Emmenanthe Benth. and Ellisia L. A third, unnamed clade comprises the genera Draperia Torr., Houellanthus (Constance) Walden & R.Patt., Hesperochiron S. Watson, and Tricardia Torr. ex S.Watson (Luebert et al. 2016; Vasile et al. 2020).

In a recent study by Vasile et al. (2021), the internal ovary architecture of Hydrophyllaceae and its evolution were studied, but the study included only three representatives of Hydrophyllinae. The focus was on the reduction versus the increase of ovule numbers across the family, and it revealed a surprising diversity of placenta types (i.e., placenta form and size and the way ovules are attached to the placenta). Placentation was found to be particularly divergent in the three species of Hydrophyllinae studied. Placentation appeared to correlate with the high variability of ovule and seed number in the clade. In the present study, we expand the sampling to explore this aspect in greater detail.

Another aspect of Hydrophyllaceae that is still incompletely studied is modifications of the stamen–corolla tube that are present in the form of often scale-shaped outgrowths, protuberances, or invaginations. They are variously termed corolla scales, faucal corolla tube modifications, or compartmentalized ovules with large anthers—such as the coronas or paracorollas of many Sileneae, Caryophyllaceae (Arber 1939; Thomson 1942), the often brightly colored scales that enclose the nectar in Nasa Weigend, Loasaceae (Weigend and Gottschling 2006), and the corolla appendages in Schwenckieae, Solanaceae (Paucar et al. 2020). These modifications have significant repercussions for floral architecture (sensu Endress 1996). In Hydrophyllaceae, stamen–corolla tube modifications have been shown to be integrated with the gynoecial nectary disc located at the base of the ovary. In several Hydrophyllaceae, especially some Hydrophyllinae, the modifications compartmentalize the flower, partitioning the nectar and leading to revolver architecture (Jetter and Weigend 2018).

The present study investigates the flower and fruit development of three additional species of Hydrophyllinae (Hydrophyllaceae), the monotypic genus Emmenanthe and two of the three species of the genus Pholistoma, which have not previously been studied in detail. We address the following questions: (i) How do the flower and fruit of these three species develop? (ii) How do the stamen–corolla tube modifications and the nectararies integrate into the general floral architecture? (iii) How does placentation in this clade evolve, and can something be learned from this aspect with regard to the fruit evolution of Boraginales?

Material and Methods

Plant Material

Floral buds of various developmental stages, anthetic and postanthetic flowers, and fruits of three Hydrophyllaceae species of Hydrophyllinae, namely, Emmenanthe penduliflora Benth. (US-0-BONN-38156), Pholistoma auritum (Lindl.) Lilia (US-0-BONN-38190), and Pholistoma membranaceum (Benth.) Constance (US-0-BONN-36930), were collected from cultivated plants at Bonn University Botanic Gardens (fig. 1). The seeds were obtained from the California Botanic Garden (formerly Rancho Santa Ana Botanic Garden). The samples were fixed in formaldehyde–acetic acid–ethanol (FAA; formaldehyde, ~2%; acetic acid, 4%; ethanol, 70%) for at least 1 wk, and the samples were subsequently prepared for scanning electron microscopy (SEM) and micro–computed tomography (µCT). Vouchers are stored at the herbarium of the Nees-Institut für Biodiversität der Pflanzen (BONN).

Scanning Electron Microscopy

Fixed samples were rinsed with ethanol (70%) three times, dissected under a stereomicroscope, and stored in ethanol (70%). For chemical dehydration, subsets of the samples were transferred into ethanol (70%) and acetic acid (4%) for at least 1 h or overnight. The samples were sequentially transferred into formaldehyde dimethyl acetal (FDA) for 2 h, FDA and acetone (1:1) for 1 h, and finally acetone for 1 h. The last step was repeated twice, and the samples were finally stored in acetone. Flowers representing different developmental stages were critical-point dried with CO2 in a critical-point dryer (CPD 020, Balzers Union, Liechtenstein) following standard protocols. Dried specimens were mounted on glass slide-covered aluminum stubs using conductive carbon cement (Leit-C, PLANO, Wetzlar, Germany). Dried corollas were directly mounted onto aluminum stubs using conductive carbon tape (25-mm Leit Tabs, PLANO) and conductive carbon cement. The mounted specimens were sputter-coated with gold (SCD 040, Balzers Union) for 1.5–3 min at 30 mA, depending on their topological complexity. The SEM images were taken at 15 kV using a Stereoscan 200 electron microscope (CAMBRIDGE, Cambridge, UK). The contrast and brightness of the images were linearly adjusted when needed using Affinity Photo 1.8 ( Serif, Nottingham, UK).

Micro–computed Tomography

Samples for micro–computed tomography (µCT) were prepared following Staedler et al. (2013). Four different FAA-fixed
developmental stages for each species (table 1) were transferred for infiltration into a solution consisting of ethanol (70%) and phosphotungstic acid (PTA; 1%) for at least 2 wk. The infiltration medium was exchanged every second day. Subsequently, the samples were dehydrated in an ascending ethanol series (80%, 90%, and 99%), each step containing PTA (1%), and finally were transferred into a solution of acetone and PTA (1%) that was exchanged three times. The samples were then critical-point dried from acetone in CO₂ (CPD 020, Balzers Union) and individually mounted on thin aluminum rods (diameter: 3 mm) using two-component epoxy glue (UHU PLUS Sofortfest, UHU, Bühl, Germany).

Analyses were carried out using a SKYSCAN 1272 μCT scanner (Bruker, Kontich, Belgium) equipped with an L11871-20 microfocus X-ray source (Hamamatsu Photonics, Hamamatsu City, Japan) and a Ximea xIRAY16 camera (Ximea, Münster, Germany). Scan settings are given in table 1. Reconstructions were performed using the InstaRecon engine version 2 (InstaRecon, Champaign, IL). The resulting image stacks were visualized using Dragonfly 2020.1 (Object Research Systems 2020). The histogram for each scanned object was manually adjusted between 5000 and 50,000. For visualization purposes, in the volume view of the reconstructed data, functions such as “shapes” and “clip-box” were used to hide distracting features, such as parts of the calyx, corolla, or anthers. “Shapes” or the “clip” function was also used to separate the individual flowers of an inflorescence tip. Screenshots of virtual longitudinal and cross sections were taken after manually adjusting the yaw, pitch, and role of the image plane. To visualize the placentae, ovules, and seeds, the “segmentation” module of the software Dragonfly was used. Regions of interest (ROIs) were segmented using a combination of histogram-based and manual segmentation. Segmented volumes were refined using the function “process islands,” with the voxel count set to 50. To further remove artifacts, the following morphological operations were applied where necessary: 2 × “dilate,” 1 × “smooth,” and 1 × “erode.” Surfaces were rendered with the “generate contour mesh” tool, and the threshold values for the ROIs were adjusted to between 5 and 50.

Results

General Development and Morphology

Flowers of Emmenanthe and Pholistoma are tetracyclic and pentameric with a bicarpellate, syncarpous gynoecium. Flowers are arranged in terminal scorpioid cymes, although complex

Fig. 1  Flowers and fruits of the studied species. A, B, Emmenanthe penduliflora. A, Anthetic flower. B, Capsule; persistent corolla and androecium removed. C, D, Pholistoma auritum. C, Anthetic flower. D, Capsule. E, F, Pholistoma membranaceum. E, Anthetic flower. F, Capsule; the fruits turn purple as they mature.
Figure 3). At anthesis, the anthers of

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**Table 1**

| Taxon, developmental stage | Source voltage (kV) | Source current (µA) | Exposure time (ms) | Frame averaging | Pictures per sample | Camera binning | Isotropic voxel size (µm) | Figure(s) |
|----------------------------|---------------------|---------------------|-------------------|----------------|---------------------|----------------|--------------------------|-----------|
| Emmenanthe penduliflora:   |                     |                     |                   |                |                     |                |                          |           |
| Inflorescence tip          | 45                  | 165                 | 444               | 6              | 1472                | 2 x 2          | 4.5                      | 5A–5D     |
| Preanthetic flower         | 45                  | 165                 | 1166              | 6              | 1438                | 2 x 2          | 1.5                      | 6A, 6B    |
| Anthetic flower            | 45                  | 165                 | 444               | 6              | 1544                | 2 x 2          | 6.3                      | 6C, 6D, 9A–9C |
| Capsule                    | 45                  | 165                 | 444               | 6              | 1537                | 2 x 2          | 7                        | 7B, 7C    |
| Pholistoma auritum:        |                     |                     |                   |                |                     |                |                          |           |
| Inflorescence tip          | 45                  | 165                 | 755               | 6              | 1401                | 2 x 2          | 4                        | 5E–5H     |
| Preanthetic flower         | 45                  | 165                 | 1166              | 6              | 1532                | 2 x 2          | 2.7                      | 6E, 6F    |
| Anthetic flower            | 45                  | 165                 | 444               | 6              | 909                 | 2 x 2          | 7.5                      | 6G, 6H, 9D–9F |
| Capsule                    | 45                  | 165                 | 444               | 6              | 1557                | 2 x 2          | 6                        | 7E, 7F    |
| Pholistoma membranaceum:   |                     |                     |                   |                |                     |                |                          |           |
| Inflorescence tip          | 45                  | 165                 | 1166              | 6              | 1485                | 2 x 2          | 3                        | 5J–5L     |
| Preanthetic flower         | 45                  | 165                 | 1166              | 6              | 1478                | 2 x 2          | 2.5                      | 6J        |
| Anthetic flower            | 45                  | 165                 | 1166              | 6              | 1240                | 2 x 2          | 3                        | 6K, 6L, 9G–9I |
| Capsule                    | 45                  | 165                 | 444               | 6              | 1472                | 2 x 2          | 4.5                      | 7H, 7I    |

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synflorescence types are usually formed. Flowers are pedicellate and are subtended by a bract and a pair of prophylls. The inflorescence tip is slightly rolled, and the flowers develop on the outer side of the spiral. Flower development is associated with an elongation and unrolling of the inflorescence axis. The flower buds develop acropetally in a zigzag line.

At the onset of floral ontogeny, soon after the hemispherical floral apex arises from the inflorescence axis, it develops into a pentagonal receptacular plate. Subsequently, the calyx, corolla, and androecium develop in centripetal order (figs. 2, 3). The sepals develop in a 2/5 spiral order (not shown) surrounding the pentagonal plate. The calyx reaches its final size before anthesis. Sepal estivation is always apert. The corolla and androecium are initiated sequentially and independently of each other (fig. 2). The petal primordia are initiated subsimultaneously as rounded edges of the pentagonal receptacular plate alternating with the sepals. They grow to almost the same size in the early stages of development (fig. 2B, 2F, 2J). The petal primordia appear subsimultaneously after the petal primordia in antesepal positions, and they either grow to the same size (fig. 2C) or have profound differences in size (fig. 2G, 2K). The petals and stamens develop at the same rate until they become basally fused and grow on a common basis (fig. 2D, 2H, 2L). Following basal fusion, the petal and stamen primordia flatten, and a first distinction between the anthers and filaments becomes apparent (fig. 3). The elongation of the corolla lobes occurs late during ontogeny, after the stamen–corolla tube is completely formed.

The sepals of Emmenanthe penduliflora elongate faster than the inner floral organs (fig. 2A–2D) and protect them up to anthesis. The calyx lobes are lanceolate, with distinct sinus appendages and large trichomes. The corolla lobes show contorted estivation (fig. 3F). The stamens are exerted and are the same height as the style. Flowers are erect.

Pholistoma membranaceum (figs. 2I–2L, 3G–3I) shows only minor differences compared with P. auritum. These are the only differences: at anthesis, P. membranaceum has small oblong calyx lobes with large trichomes and without sinus appendages.

The stamens are exerted and surpass the style.

**Gynoeicum and Nectary**

The gynoeicum is initiated when the petals and stamens begin to bend over the floral apex. At this point, a depression is formed at the center of the floral apex (fig. 2D, 2H, 2L). The walls of the two young horseshoe-shaped carpels form a ring at the beginning of gynoeicum development (fig. 3A, 3B, 3D, 3G). The carpel primordia are fused from the onset of their development and grow apically to form a gynoeical cone (fig. 4A, 4E, 4F). The growth of the tips of the carpels slightly exceeds the growth of the fused walls so that the resulting gynoeical cone is bifid at the tip.

After formation of the gynoeical cone, the two carpel lobes elongate to form a bifid style with two separate stigmata (fig. 4B, 4F, 4J). Simultaneously, the ovary is differentiating, and its basal part starts to flatten. The flattening of the ovary base demarcates the onset of nectary development. Specifically, at the very bottom part of the ovary, five small protrusions appear, usually with one nectarostoma in an antepetalous position. These five protrusions gradually develop into nectary lobes, and more nectarostoma appear on their surface (fig. 4B, 4F, 4J).

In E. penduliflora, the gynoeical cone is oblong and exceeds the developing anthers in height (figs. 4A, 5B). During the preanthetic developmental stages, the ovary is ovoid and elongates to gradually become oblong at anthesis (fig. 4C, 4D). Large glandular and filiform trichomes appear on the ovary surface during development (fig. 4C). The nectary disk enlarges and becomes a distinct hypogynous disk at anthesis (fig. 4C, 4D). It slightly exceeds the circumference of the ovary at its widest point (figs. 4D, 6D). The style splits into two short branches in the
Fig. 2  Early flower development shown in successive developmental stages of the studied species, analyzed using scanning electron microscopy. 
A–D, Emmenanthe penduliflora. A, Calyx initiation. B, Corolla initiation. C, Androecium initiation. D, Gynoeceum initiation. E–H, Pholistoma auritum. E, Calyx initiation. F, Sequential corolla and androecium initiation. G, Petal and staminal primordia develop subsimultaneously. H, Gynoeceum initiation. I–L, Pholistoma membranaceum. I, Calyx initiation. J, Sequential corolla and androecium initiation. K, Petal and staminal primordia develop subsimultaneously. L, Gynoeceum initiation. ca = calyx; co = corolla; gy = gynoeceum; st = stamen. Scale bars = 100 μm (A–H), 80 μm (I–L).
early developmental stages (fig. 4B). As they elongate, they bend away from each other (fig. 4C). The distinct subglobose stigmata with a papillate surface develop relatively late (fig. 4C, 4D). At anthesis, the style branches are one-third of the style length, and scattered filiform trichomes cover the style (fig. 4D).

*Pholistoma auritum* has a gynoecial cone half the size of the developing anthers (figs. 4E, 5F). The conical ovary increases in diameter and retains its shape throughout development. Large filiform trichomes first develop at the upper part of the ovary (fig. 4F, 4G), and later, shorter trichomes densely cover the
Fig. 4  Gynoecium development shown in successive developmental stages of the studied species analyzed using scanning electron microscopy. A–D, *Emmenanthe penduliflora*. A, Gynoecial cone. B, Ovary formation and nectary disk initiation. C, Ovary enlargement, style elongation, stigmatic papillae, and nectary disk development. D, Anthetic gynoecium; close-ups of the stigmatic head (top right) and of a nectary lobe (bottom right). E–H, *Pholistoma auritum*. E, Gynoecial cone. F, Ovary formation and nectary lobe initiation. G, Ovary enlargement, style elongation, stigmatic papillae, and nectary ring development. H, Anthetic gynoecium; close-ups of the stigmatic head (top right) and of a nectary lobe (bottom right). I–L, *Pholistoma membranaceum*. I, Gynoecial cone. J, Ovary formation and nectary disk initiation. K, Ovary enlargement, style elongation, stigmatic papillae, and nectary disk development. L, Anthetic gynoecium; close-ups of the stigmatic head (top right) and of a nectary lobe (bottom right). The arrows point to nectarostomata. an = anther; c1 = carpel 1; c2 = carpel 2; nd = nectary disk; nl = nectary lobe; o = ovary; s = style; sp = stigmatic papillae; st = stamen. Scale bars = 100 μm (A, B, E, F, I, J), 200 μm (C, D, G, H, K, L).
Fig. 5  Inner gynoecium development of the studied species, analyzed using micro–computed tomography, very early stages. A–D, Emmenanthe penduliflora. A, B, Gynoecial cone formation; onset of placenta and ovule development. A, Virtual cross section. B, Virtual longitudinal section. C, D, Ovule development. C, Virtual cross section. D, Virtual longitudinal section. E–H, Pholistoma auritum. E, F, Gynoecial cone formation; onset of placenta and ovule development. E, Virtual cross section. F, Virtual longitudinal section. G, H, Ovule development. G, Virtual cross section. H, Virtual longitudinal section. I–L, Pholistoma membranaceum. I, J, Gynoecial cone formation; onset of placenta and ovule development. I, Virtual cross section. J, Virtual longitudinal section. K, L, Ovule development. K, Virtual cross section. L, Virtual longitudinal section. Dashed lines with letters indicate the corresponding virtual sections in other panels of this figure. an = anther; c1 = carpel 1; c2 = carpel 2; ca = calyx; co = corolla; nd = nectary disk; ov = ovule; pl = placenta; s = style; se = septum; st = stamen. Scale bars = 100 μm.
Fig. 6  Inner gynoecium development of the studied species, analyzed using micro–computed tomography, preanthetic and anthetic developmental stages. A–D, Emmenanthe penduliflora. A, B, Preanthetic gynoecium. A, Virtual cross section. B, Median virtual longitudinal section through the carpels. C, D, Anthetic gynoecium. C, Virtual cross section. D, Virtual longitudinal section. E–H, Pholistoma auritum. E, F, Preanthetic gynoecium. E, Virtual cross section. F, Median virtual longitudinal section through the carpels. G, H, Anthetic gynoecium. G, Virtual cross section. H, Virtual longitudinal section. I–L, Pholistoma membranaceum. I, J, Preanthetic gynoecium. I, Virtual cross section. J, Median virtual longitudinal section through the carpels. K, L, Anthetic gynoecium. K, Virtual cross section. L, Virtual longitudinal section through the carpels. Dashed lines with letters indicate the corresponding virtual sections in other panels of this figure. an = anther; ca = calyx; co = corolla; nd = nectary disk; ov = ovule; pl = placenta; s = style; se = septum; st = stamen. Scale bars = 100 μm.
remaining surface and the base of the style. As the ovary enlarges, a ring-shaped nectary disk is formed and becomes distinct at anthesis (figs. 4G, 4H, 6H). The five lobes slightly protrude at anthesis and can be distinguished only by the presence of the nectarostoma, which are limited to the tip of each lobe (fig. 4H). The subglobose papillate receptive surface of the stigmaticum appears early during development (fig. 4F). The style of P. auritum significantly elongates shortly before anthesis. At this stage, it is covered with scattered trichomes. The style cleft is in the upper third-quarter of the style, and the two stigmata slightly separate from each other at anthesis (fig. 4H).

Pholistoma membranaceum has a gynoecial cone almost the same size as the developing anthers (figs. 4I, 5f). In some specimens of P. membranaceum, one carpel is slightly larger than the other one, indicating potential sequential insertion, but with a very short plastochron. This difference in size remains visible until anthesis, as very often one style branch is shorter than the other one (fig. 4K, 4L). The initially conical ovary (fig. 4f) increases its diameter while it continues to grow to reach its final subglobose shape (fig. 4f–4L). At anthesis, the ovary wall significantly bulges at the line of carpel unification. Scattered, large, scabrid trichomes with an extensive base gradually develop on the ovary surface (fig. 4f–4L). The nectary disk is initially flattened and has a notably wider diameter than the rest of the young ovary (fig. 4f). It grows until it forms a cup-shaped disk with an undulate pentagonal circumference (fig. 4K, 4L). The stigma formation on the carpel apexes starts early with the development of papillate tissue (fig. 4f) and later forms a subglobose stigmatic head. The bifid style mostly elongates at later developmental stages, and the style cleft is formed in the upper third of the style’s length (fig. 4f, 4K, 4L).

**Internal Ovary Architecture**

At the onset of gynoecial cone development, two placentae develop through contraction of the septa to the periphery (fig. 5A, 5B, 5E, 5F, 5I, 5J). Placentation is parietal. Subsequently, internal ovary architecture development is largely affected by ovule number and placentation. A compitum is always present in the style.

In E. penduliflora (figs. 5A–5D, 6A–6D), after the gynoecial cone closes, the placenta and a basal as well as an apical septum are differentiated, and the first ovule primordia become apparent (fig. 5A, 5B). This species is pluriovulate (i.e., it has more than four ovules), and the intrusive parietal placenta with true septa form two compartments in the ovary as they develop. At anthesis, there are usually three and sometimes four layers of ovules, all of which develop almost simultaneously (figs. 5B, 5D, 6B, 6D). Each placenta ends up having two rows of ovules and in total six to eight ovules arranged in three or four layers, respectively (fig. 6C, 6D). The ovules of one layer are not at the exact same level, resulting in a zigzag arrangement of the two rows of ovules of each placenta (fig. 6D). The mature placentae have a triangular shape in cross section (fig. 6D). Ovule orientation is epitropous-ventral.

In P. auritum (figs. 5E–5H, 6E–6H), the placenta as well as short basal and apical septa are already differentiated once the gynoecial cone closes (fig. 5E, 5F). The lateral parts of the young placentae flatten right after initiation and, together with the septa, form an initially T-shaped structure in cross section. As the ovary enlarges, four round ovules, two per placenta, start to develop (fig. 5E–5H). The ovules of each placenta alternate as a result of mutual displacement and a lack of space in the central compartment; however, all four ovules belong to a single layer. This ovule alternation results in a different ovule orientation (fig. 6F). The lowermost ovule of each placenta becomes epitropous-ventral, whereas the uppermost becomes hypotropous-ventral. The placentae gradually enlarge, create one central compartment, start to line the ovary wall, expand around and between the ovules, and almost completely fill the ovary at anthesis (fig. 6E–6H).

Pholistoma membranaceum (figs. 5I–5L, 6I–6L) has a developmental trajectory identical to that of P. auritum. The main differences are that the placentae are smaller, creating, as a result, a slightly larger central compartment, and that the ovules are not perfectly rounded.

**Fruit**

After anthesis, the ovary, placentae, and fertilized ovules gradually enlarge until fruit maturity. The fruit is a loculicidal capsule with two locules. The style persists on one valve of the opened capsule.

Emmenanthe penduliflora (figs. 1B, 7A–7C) retains all of its floral organs, including the corolla and stamens, until fruit maturity. The pedicel doubles in length, and the withering, papery, marcescent corolla encloses the capsule. The nectary disk retains its shape intact at fruit maturity (fig. 7A). The two placentae enlarge and occupy most of the internal volume of the oblong capsule. The seeds are flat and slightly concave. They are spirally arranged around the placentae (fig. 7B, 7C). Fruits are nodding at maturity (fig. 1B).

In P. auritum (figs. 1D, 7D, 7E), the entire stamen–corolla tube wilts and falls off after anthesis. The nectary disk becomes part of the pericarp (fig. 7D). The placentae completely fill the fruit (fig. 7E). Fruits are globose to ovoid and are enclosed by the persistent accrescent calyx (figs. 1D, 7D, 7E). The capsules have a light green color with red spots (fig. 1D) until they are completely dry and open. Seeds are brown and round in shape, and the seed coat surface is moderately reticulate with polygonal cavities. There are usually four or fewer mature seeds.

The fruit development and morphology of P. membranaceum (figs. 1F, 7F–7H) are similar to that of P. auritum. The large nectary disk creates a platform at the fruit base. The accrescent calyx persists but does not enclose the mature fruit (fig. 1F). Fruits are initially light green in color but turn purple as they mature (fig. 1F). The dry opened capsules retain a deep purple color. Seeds are ovate in shape, and the seed coat surface is moderately reticulate with polygonal cavities. There are four or, usually, fewer mature seeds.

**Stamen–Corolla Tube Modifications and Floral Synorganization**

Ten modifications develop on the adaxial side of the stamen–corolla tube. Their development starts during late flower ontogeny, after the differentiation of the stamens into well-defined anthers and filaments and of the gynoecium into young stigmata, style, and ovary with a nectary disk. The modifications, the corolla tube itself, the filaments, and the style subsequently
**Fig. 7** Mature capsules of the studied species. 

A–C, *Emmenanthe penduliflora*. A, Scanning electron microscopy (SEM) image; outer view of a closed capsule, dehiscence line visible. B, Micro-computed tomography (μCT)-based volume rendering showing seed arrangement; lateral view (looking at the line of carpel fusion). C, μCT-based volume rendering showing seeds and placentae; view of one carpel (looking at the dehiscence line). D–F, *Pholistoma auritum*. D, SEM image; outer view of a closed capsule, dehiscence line visible. E, μCT-based volume rendering showing seeds and placentae; only half of the placenta is displayed because it completely lines the capsule and the ovules would otherwise be obscured; lateral view. F, μCT-based volume rendering showing seed arrangement; vascular bundles connecting seeds to placentae visible; view of one carpel. G–I, *Pholistoma membranaceum*. G, SEM image; outer view of a closed capsule; dehiscence line visible. H, μCT-based volume rendering showing seeds and placentae; only half of the placenta is displayed because it completely lines the capsule and the ovules would otherwise be obscured; lateral view. I, μCT-based volume rendering showing seeds and placentae; only half of the placenta is displayed because it completely lines the capsule and the ovules would otherwise be obscured; view of one carpel. In B, C, E, F, H, and I, the pericarp is reconstructed and displayed with low opacity and solidity. Seeds are displayed in yellow and placentae in blue. nd = nectary disk. Scale bars = 1 mm.
elongate and are elaborated in parallel. This pattern is found across all three studied species.

In *E. penduliflora* (figs. 1A, 8A–8C, 9A–9C), the modifications are initiated as elongated protrusions neighboring the base of the filaments (fig. 8A). They retain their overall shape and only slightly increase in size during development and the elongation of the basal part of the corolla tube (fig. 8B). At the stage when the filaments have reached their final size, the modifications

![Fig. 8](image_url)  
Fig. 8  Development of stamen–corolla tube modifications of the studied species, analyzed using scanning electron microscopy. A–C, *Emmenanthe penduliflora*. A, Very young flower. B, Preanthetic flower. C, Anthetic flower. D–F, *Pholistoma auritum*. D, Very young flower; stamen removed. E, Preanthetic flower. F, Anthetic flower. G–I, *Pholistoma membranaceum*. G, Very young flower. H, Preanthetic flower. I, Anthetic flower. Asterisks indicate the modifications. st = stamen. Scale bars = 100 μm.
Fig. 9  Stamen–corolla tube modifications of the studied species, micro-computed tomography–based surface renderings and virtual sections. A–G, Emmenanthe penduliflora. A, Surface rendering of the flower viewed from the top and slightly tilted, nectary disk in green. B, Virtual cross section at the level of the nectary disk. C, Virtual longitudinal section. D–F, Pholistoma auritum. D, Surface rendering of the flower viewed from the top, nectary disk in green. E, Virtual cross section at the level of the nectary disk. F, Virtual longitudinal section. G–I, Pholistoma membranaceum. G, Surface rendering of the flower viewed from the top, nectary disk in green. H, Virtual cross section. I, Virtual longitudinal section. Dashed lines with letters indicate the corresponding reconstructed sections in other panels of this figure. Asterisks indicate the modifications. an = anther; ca = calyx; co = corolla; nd = nectary disk; nl = nectary lobe; ov = ovule; pl = placenta; s = style; st = stamen. Scale bars = 500 μm.
protrude toward and touch the base of the filaments (fig. 8C). At anthesis, the stamen–corolla tube modifications are small and rudimentary and do not form any compartments (fig. 9A–9C). They are found at the level where the nectary disk initiates (fig. 9B). The nectary lobes are found in an antepetalous position and protrude between the rudimentary modifications. The filaments are fused to the base of the corolla tube and between the lobes of the nectaries (fig. 9C). The anthetic corolla is white to yellow and tubular to campanulate (fig. 1A). The corolla tube is three times the length of the free corolla lobes. Trichomes are found only across the midvein on the abaxial side of the petals.

Development of the modifications of *P. auritum* starts as elongated ridges. In the beginning, they are associated with the base of the neighboring filament (fig. 9D). As the modifications elaborate, simple trichomes emerge along their margins (fig. 9E) and later also on the corolla tube surface between the modifications, behind the filaments (fig. 7F). The apical part of the modifications, where the filaments diverge from the corolla tube, gradually widens more than the basal part. At anthesis, each pair of modifications is present in the form of wing-shaped protuberances (figs. 8F, 9D–9F). This shape results in the enclosure of a broad and deep groove between neighboring modifications. The narrowest part of the groove is where it touches the nectary disk (figs. 8F, 9D–9F). This groove is in an antepetalous position, the same position as the nectary lobes. The anthetic corolla is purple, and a distinct dark purple ring is formed at the pale entrance to the corolla tube (fig. 1C). The stamen–corolla tube modifications are deep purple with a yellowish line on top. The white trichomes of the neighboring modifications are intertwined and, as a consequence, completely cover the grooves between the modifications. The corolla is cyathiform, and the lobes are twice as long as the corolla tube.

In *P. membranaceum*, the modifications are initiated as two shallow lobes above the insertion point of the filaments (fig. 8G). As they develop, they elongate and swell at the same stage that the filaments become free (figs. 8H, 8I, 9I). During development, the lower part of the corolla tube invaginates in the position of the modifications. Additional protrusions on the invaginations are absent (fig. 9H). At anthesis, neighboring modifications touch or almost touch each other (figs. 8G, 8I), thus forming a narrow canal above the nectary lobes. The irregular shape of the large nectary disk is due to the modifications touching the disc. The shape of the anthetic corolla is similar to that of *P. auritum* but smaller overall (fig. 1E). The white corolla has purple midveins but its lobes that lead toward the narrow canals created by the modifications (fig. 1E).

**Discussion**

In the present study, we describe the general flower and fruit development of *Emmenanthe* and *Pholistoma*, two genera of Hydrophyllaceae within Hydrophyllaceae. This is the first ontogenetic survey of overall flower development in this family. There have been few floral ontogenetic studies of Hydrophyllaceae, and they have largely concentrated on gynoecial development (Payer 1857; Hofmann 1999; Vasile et al. 2021) or on stamen–corolla tube modifications (Hofmann 1999; Jeiter and Weigend 2018). Other studies have focused on the floral morphology and anatomy of various species of Hydrophyllaceae (e.g., Berg 1985, 2009; Chuang and Constance 1992; Di Fulvio et al. 1999). The floral ontogeny of *Emmenanthe* and *Pholistoma* seems to be similar to that of other species in the family (Hofmann 1999; Vasile et al. 2021). There are minor differences among the species studied here, in particular with regard to sepal and gynoecial development and morphology, and there are also major differences in the gynoecial nectar disk and stamen–corolla tube modifications.

Gynoecial nectar disks of Hydrophyllaceae often form separate glands (e.g., *Hydrophyllum*, *Nemophila*; Jeiter and Weigend 2018; Vasile et al. 2021). The species studied here have fully developed nectary disks distinct from the base of the ovary. However, nectarostomata are restricted to lobes of the disk, as is typical of Hydrophyllaceae (Jeiter and Weigend 2018). Stamen–corolla tube modifications in Hydrophyllaceae share a highly conserved developmental trajectory (Jeiter and Weigend 2018), and the species studied here are no exception to this. Modifications develop late during floral ontogeny, and they originate from both the corolla tube and the filament bases; their number always appears to be 10. In the present study, we were able to document the presence of modifications in *Emmenanthe penduliflora*, which had previously been described as lacking modifications (Hofmann et al. 2016). The pattern of initiation and the subsequent truncation of their development have been observed in other species supposedly lacking modifications (e.g., *Draperia syzygii*; Jeiter and Weigend 2018). The presence of 10 modifications of the stamen–corolla tube appears to be the plesiomorphic condition for the family, and crucial aspects of the divergent floral morphologies of the family appear to go back at least partly to the truncation of scale development—that is, heterochronous shifts.

The predominant type of stamen–corolla tube modifications of Hydrophyllaceae are protuberances (Hofmann 1999; Jeiter and Weigend 2018). However, surprisingly, in *Pholistoma membranaceum* invaginations are found where modifications are initiated, as in all other species, but further development is dominated by the invagination of the basal part of the corolla tube.

The flower architecture of Hydrophyllaceae can thus be shown to follow broadly uniform patterns, with minor developmental shifts explaining different mature morphologies. Conversely, our study demonstrates surprisingly divergent internal ovary architectures. As shown previously, seed numbers are highly variable in Hydrophyllaceae and largely depend on changes in internal gynoecium development (Vasile et al. 2021). Here, we focus on another highly variable aspect: placentation. Placentation in Hydrophyllaceae is originally parietal or intrusively parietal. However, various changes in developmental trajectories result in profoundly different internal ovary architectures as well as changes in placentation. These changes also co-occur with changes in ovule and seed number, shape, size, and orientation. Figure 10 summarizes our current understanding of the internal ovary architecture of Hydrophyllaceae (Di Fulvio et al. 1999; Hofmann 1999; Vasile et al. 2021). On the basis of the ancestral character state reconstruction of Vasile et al. (2021), we propose hypothetical character states for each node of the phylogeny (fig. 10). An ovary with four rows of ovules (two per placenta) in several tiers can be found in all three clades of the family (fig. 10; type I or II). We propose that this represents the ancestral character state of the family. We also suggest that the multiplication of ovule rows per placenta has independently taken place at least
twice within Romanzoffeae (fig. 10; type III). Accordingly, the simple truncation of ovary development following the initiation of the first layer of ovules (Vasile et al. 2021) must have resulted in descendants with only the four ovules of the first layer (paedo- morphosis; fig. 10; type IV), probably explaining the multiple transitions in Hydrophyllae toward a reduction in ovule number to four (Vasile et al. 2021). In Hydrophyllae, this is also associated with the development of unilocular ovaries and the inclusion of the ovules between the enlarged placentae. Specifically, in Hydrophyllum, the placentae completely fill the ovary (fig. 10; type VII), leaving no free space. Conversely, the placentae of Pholistoma and Nemophila leave more free space in the central locule (fig. 10; types VI, VIII). This probably permits higher plas- ticity and opens the pathway to secondary multiplications of the ovule layers during gynoecium development. In Pholistoma, as well as Nemophila, more than four ovules are common (Berg...
tion is slightly delayed, as our data on
seems to be their heterochronous development: lateral parts of
1985; Chuang and Constance 1992; Vasile et al. 2021; type VIII). The key factor for the evolution of unilocular ovaries
family observed in Emmananthel to the center of the ovary.
formation of flattened placentae on which ovules develop on
both surfaces in the closely related Eucrypta chrysanthemifolia
(Benth.) Greene (Hofmann et al. 2016; Vasile et al. 2021) thus
must have evolved only once within the family (fig. 10; type V),
and whether this species is a descendant of a four-ovulate or plu-
riovulate ancestor remains enigmatic.

In addition to the reduction of ovule numbers and a fixed
number of four ovules per ovary, placentalation is another crucial
factor in the evolution of mericarpic and endomeracarpic fruits
in Boraginales. All capsular fruiting families across Boraginales
have parietal or intrusive parietal placentalation (Weigend and
Hilger 2010; Hilger and Weigend 2016; Hofmann et al. 2016;
Jeiter et al. 2016), whereas the remaining nondcapsular fruiting
families show basal to axile placentalation. Here, the ontogenetic
data on the internal ovary architecture show that changes in de-
velopmental rate or timing (heterochrony) can cause profound
changes in otherwise very conserved morphologies. Hetero-
chrony has also been suggested as the main mechanism that
led to the reduction to four ovules (Vasile et al. 2021). The data
presented here underscore a surprisingly high degree of conser-
vation in the structure and development of Hydrophyllaceae—despite
the obvious differences in flower morphology across the family.
In this taxon, functional floral diversity is realized by simple
shifts in the relative development of different organs. At a first

1985; Chuang and Constance 1992; Vasile et al. 2021; fig. 10;




glance, this is in stark contrast with the considerable morpho-
logical diversity of the nectaries, internal and external ovary ar-
chitecture, and ovule numbers and arrangements observed in
the Hydrophyllaceae. These characters are largely conserved
amongst most families in Boraginales and indeed are sufficiently
conserved across many groups of angiosperms to have been clas-
sically used in higher-order classification. Flower and fruit on-
togeny and evolution in Boraginales are thus surprisingly com-
plex phenomena, and recent progress with contrast-enhanced
\(\mu CT\), 3D visualization, and an expanded sampling provides
novel insights into flower evolution across this order. Our data,
however, indicate that minor heterochronous shifts in individual
aspects of the developmental trajectories—placentalation, ovule
initiation, ovary expansion—are able to explain the diversifica-
tion of the internal and external ovary architectures of Hydro-
phyllaceae. An expansion of these studies to the few morpholo-
ically aberrant lineages that so far have not been studied—for
example, Hoplestigmataceae and Lennoaceae—promises a
deeper understanding of flower and fruit evolution across the en-
tire order’s lineage.

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