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Widespread and homologous life–history traits within the Rhopalodiales (Bacillariophyceae)

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Abstract: Significant changes have been proposed for the order Rhopalodiales, including subsuming *Rhopalodia* O.Müll. into *Epithemia* Kütz. to maintain monophyly and recognition of the genus *Tetralunata* Hamsher et al. However, hesitancies about adopting these higher–level classifications remain. We report widespread and homologous internal valve and life history traits as evidence to guide higher–level classification within the Rhopalodiales. We provide the first reports of internal valve formations in five species and show examples from six species representing multiple branches in the Rhopalodiales: *Epithemia (Rhopalodia) gibba* (Ehrenb.) Kütz., *Epithemia adnata* (Kütz.) Bréb., *Epithemia turgida* (Ehrenb.) Kütz., *Epithemia argus* (Ehrenb.) Kütz., *Epithemia catenata* Schvarcz, Stancheva et Steward, and *Epithemia reicheltii* Fricke. This observation, along with shared, unique cell development and life history traits including endosymbiosis and sexual reproduction, should be considered in phylogenetic revisions.

Keywords: diatoms, *Epithemia*, *Innenschalen*, internal valves, life history, *Rhopalodia*, spheroid bodies

Introduction

Recent multigene phylogenies have explored relationships within the Rhopalodiales and Surirellales – groups characterized by raphe systems that open internally to a siliceous canal – and resulted in significant proposed changes in genus–level classification within these orders (Ruck et al. 2016b). Among the broader relationships resolved from analysis of plastid rbcL and psbC, mitochondrial cob, and nuclear SSU rDNA and partial LSU rDNA genes, was *Epithemia* nested within a paraphyletic *Rhopalodia* O.Müll., with *Epithemia* Kütz. having nomenclatural priority for a broader monophyletic group (Ruck et al. 2016b). Support for the molecular phylogenies included broad morphological homologies in valve symmetry, raphe placement, and silicification patterns. The revised classification has been adopted by many researchers (Ruck et al. 2016a; Jahn et al. 2017; Kamakura et al. 2021; Spaulding et al. 2021); however, subgeneric groups have been proposed to accommodate previously recognized genera (Cocquyt et al. 2018) that may represent potentially monophyletic groups within the broader Rhopalodiales, yet critical hesitancies remain among researchers in adopting the new classifications and in the justification for interpreting molecular phylogenies for higher–level classification (Rybak et al. 2020; Vigneshwaran et al. 2021; Kociolek et al. 2024b,c). Other recent phylogenic analyses identify similar paraphyletic *Rhopalodia* taxa (Kociolek et al. 2024c) as Ruck et al. (2016), whereas Schvarcz et al. (2022; SSU (18S rRNA), psbC, and rbcL genes) and Moulin et al. (2024; 18S rRNA, psbC, and rbcL genes) introduce further paraphyly in the broader group's phylogeny with additional marine taxa. Here we report new observations on life history traits among the Rhopalodiales that, together with other cellular and reproductive traits, may lend further support in recognizing natural groups within the Rhopalodiales.

Several life history strategies for dormancy or responses to inimical conditions are found across the diatoms and offer potential as homologous life history traits in support of higher–level classifications (e.g., Sims et al. 2006; Mizuno 2008; Kaczmarska & Ehrman 2021). Resting spores are most common in coastal centric or multi–polar lines (Hargraves 1983), although several freshwater taxa and *Eunotia* Ehrenb. also have this perennation strategy (Von Stosch & Fecher 1979; Edlund & Stoermer 1993; Edlund et al. 1996). Resting cells, a physiological rather than morphological dormancy strategy, are especially common among planktonic forms (Sicko–Goad et al. 1989). More common in the araphid and raphid lineages are craticula (Schmid 2009) and

internal valves (*Innenschalen;* Geitler 1980), which are typically a cellular response to osmotic shifts or environmental stress.

Internal valves (*Innenschalen*, in part *Häutungen*) are produced via mitotic and silicification pathways as part of the vegetative phase in the diatom life history. Geitler (1980) presented three mitotic mechanisms that are used to produce internal valves in the pennate diatoms; they appear to be the mechanisms shared among multiple lineages that produce internal valves (Hustedt 1927; Geitler 1927 [as *Häutung(en)*], 1970, 1971, 1980; Thaler 1972). The three internal valve pathways vary both in how nuclei and organelles are allocated to the resulting internal and diminutive (or vestigial) cells and in how the internal valves are produced in those cells (Kaczmarska et al. 2013). Internal valves are often more heavily silicified than normal diatom valves. They are also typically arched or rounded in shape, which is controlled by the internal structure of the parent cell and/or by turgor pressures within the internal cell membrane, rather than by sibling valve formation and complementarity as in normal mitosis and valve morphogenesis (Round et al. 1990).

Among the araphid and raphe–bearing lineages, internal valves have been identified in many taxa, including *Fragilariforma* D.M.Williams et Round (Howard & Morales 2012), *Meridion* C.Agardh (Geitler 1971; Stancheva 2006), *Achnanthes* Bory (Geitler 1980), *Eunotia* (Hustedt 1927; Stancheva 2006), *Gomphonema* Ehrenb. (Kociolek & Stoermer 1991), *Anomoeoneis* Pfitzer (Müller 1899; Geitler 1927; Kociolek & Herbst 1992), *Nitzschia* Hassall (Krasske 1927; Liebisch 1929; Kociolek & Herbst 1992), *Hantzschia* Grunow (Loescher 1972; Geitler 1980) and *Pinnularia* Ehrenb. (Edlund & Stoermer 1997). Within the groups with raphe systems that open internally to a siliceous canal, the genera *Entomoneis* Ehrenb. (as *Amphiprora* Ehrenb., Geitler 1970; as *Stauronella* Mereschk., Liebisch 1929), *Rhopalodia* (*R. musculus* (Kütz.) O.Müll., Krasske 1927; Liebisch 1929; *R. gibberula* var. *sphaerula* O.Müll., Müller 1899), *Epithemia* (*E. zebra* v. *saxonica* (Kütz.) Grunow, Thaler 1972; *E. argus* (Ehrenb.) Kütz., *E. reticulata* Nägeli ex Kütz., *E. hyndmannii* W.Sm., Sims 1983; *E. alpestris* Kütz., Kociolek et al. 2024c; *E. schoemanii* Kociolek, Hamsher et Taylor; Kociolek et al. 2025b), *Tetralunata schoemanii* Kociolek et al. (Kociolek et al. 2024a), *Surirella* Turpin (*S. ovata* Kütz.; Krasske 1927), and *Campylodiscus* Ehrenb. ex Kütz. (as *Surirella fastuosa* (Ehrenb.) Ehrenb., Liebisch 1929) produce internal valves.

As part of efforts to study raphid diatoms with cyanobacterial endosymbionts (spheroid bodies), combined with efforts to explore diatom diversity in natural habitats rich in canal raphe–bearing taxa, we investigated cultures and natural collections of members of the Rhopalodiales to identify homologous life history traits. Specifically, we report on homologous Type 3 internal valve formation (Geitler 1980) in *Epithemia* (*Rhopalodia*) *gibba* (Ehrenb.) Kütz., *E. adnata* (Kütz.) Bréb., *E. turgida* (Ehrenb.) Kütz., *E. argus* (Ehrenb.) Kütz., *E. catenata* Schvarcz, Stancheva et Steward, and *E. reicheltii* Fricke, including the first report of internal valve formation in five of these taxa. Geitler's Type 3 process was referred to as *Häutung(en)* or "molting" when originally described for *Anomoeoneis sculpta* (Ehrenb.) Cleve (Geitler 1927). Cholnoky (1928) quickly responded that this process is internal valve formation rather than molting, and Geitler (1980) eventually presented the *Häutung(en)* process as one type of internal valve formation, which he described as Type 3 *Innenschalen*, a process based on cytology carefully described by Thaler (1972). In some instances, additional acytokinetic mitoses also occur to produce multiple valves called supernumerary valves as part of the internal valve cytological sequence (Kaczmarska et al. 2013). We will use the term "Type 3 internal valve formation" to describe this process; however, the term *Häutung(en)* has seen continued use to describe internal valve formation in the Rhopalodiales (e.g., *Epithemia*, Sims 1983; Kociolek 2025b; *Tetralunata*, Kociolek 2024a).

Materials and Methods

Recent efforts to culture canal raphe diatoms with nitrogen–fixing cyanobacterial endosymbionts produced many cultures from the Rhopalodiales representing the subgenera *Epithemia* and *Rhopalodia* from habitats in western Montana, USA (Abresch et al. 2021, 2024). Cultures and growth conditions are as described in Abresch et al. 2024. A culture of *E. catenata* UHM3211 was provided by Grieg Steward (University of Hawaii at Manoa) and grown using media and conditions in Schvarcz et al. (2022). Images of live culture material were gathered from wet mounts using a Leica Model DM E, 40× C PLAN objective, N.A. 0.65, and a Google Pixel 3 imaging system.

Natural collections of Rhopalodiales were also examined for evidence of internal and supernumerary valve formation and life history strategies. Images were gathered using an Olympus BX51 outfitted with differential interference contrast, an oil–immersion PlanApo 1000× lens (numerical aperture, NA 1.40), and an Olympus QImaging 5.0 system. Material examined included:

SMM–MBE2650a, Lake Superior, Cook County, near Grand Marais, Minnesota, USA (23 September 2021), epilithic collections from 4 m water depth from Lake Superior, coll: MBE;

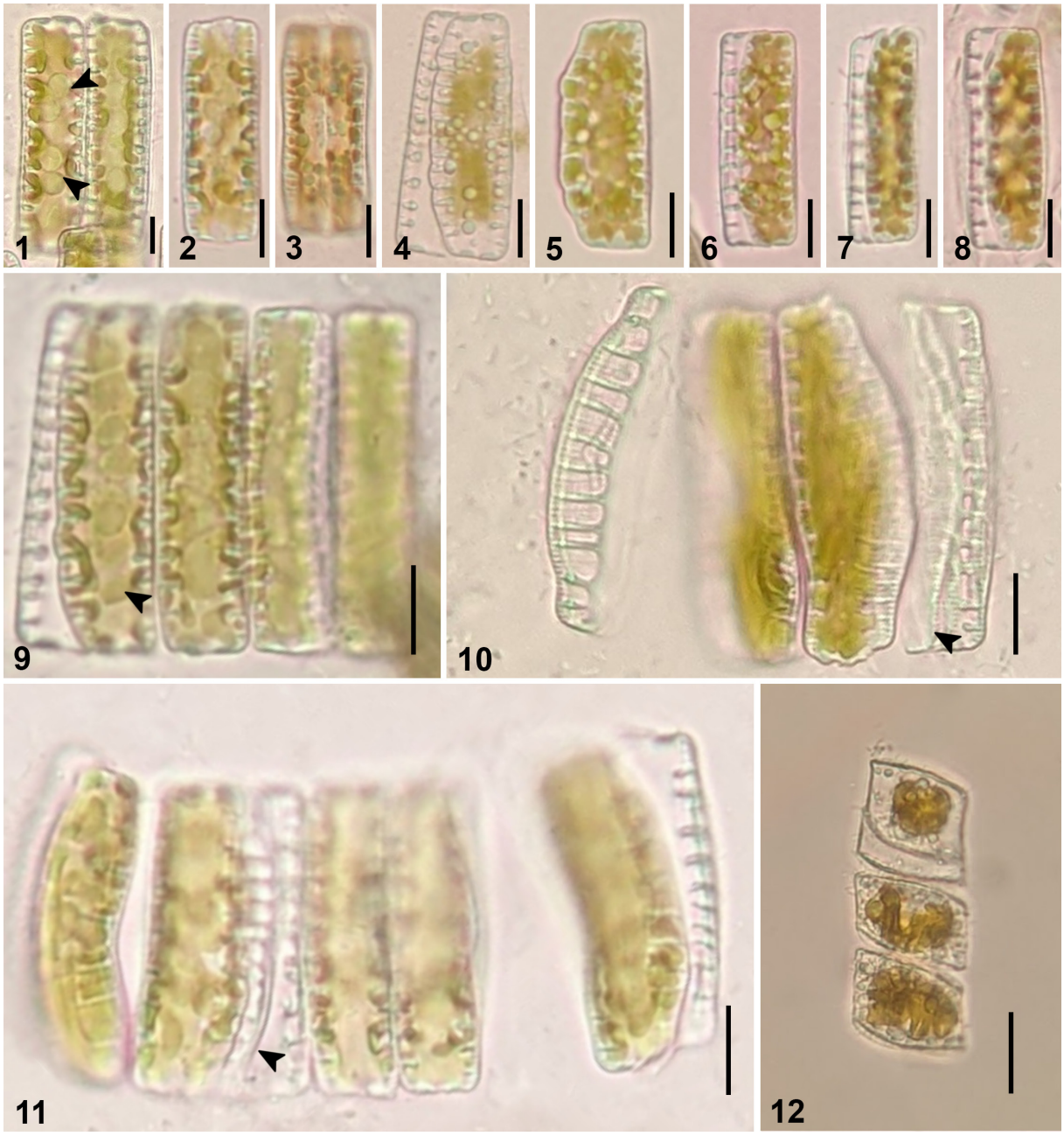
SMM–MBE1285a, Fishtrap Lake, Morrison Co., Minnesota, USA (16 May 1997), periphyton on boat landing, coll: MBE–diverse hard water flora;

SMM–M87, Lake Hovsgol, Hatgal, Mongolia (12 June 1996), oil docks N of town, S side of pier in two shallower quiet areas, *Ulothrix* Kütz. in 0.1 m depth, coll: MBE, associated taxa *Epithemia*, *Didymosphenia* M.Schmidt, *Pinnularia*, *Cocconeis* Ehrenb., *Rhopalodia*.

Additional material containing sexually reproducing cells of Rhopalodiales was examined from natural periphyton collections of *Epithemia (Rhopalodia) gibba* from Silver Lake Fen, Iowa, USA (July 1995; Leica Dialux, 400× PlanApo oil immersion, NA 1.4, Kodachrome 25) and *Epithemia turgida* from West Lake Okoboji, Iowa, USA (May 2017; material preserved in 2% glutaraldehyde, mounted in Karo syrup (Transeau's medium; Stevenson 1984) imaged with an Olympus BH2, oil immersion 100× S Plan lens, NA 1.25 and Nikon Coolpix 995 camera).

Results

During the course of routine culture maintenance and examination, samples of *Epithemia adnata*, *Epithemia catenata* and *Epithemia* (*Rhopalodia*) *gibba* shifted from normal vegetative growth and mitoses (Figs 1–3, 13) and were seen to produce Type 3 internal valves (Figs 4–12, 14–16), especially in older cultures. Type 3 internal valve formation starts with an acytokinetic mitosis, with the resulting nuclei and all chloroplasts contained in the internal cell; a small diminutive or vestigial area remains between the internal valve and the original valve but contains no visible cytoplasmic contents (Figs 4–9, 12) or new valves. One of the nuclei in the internal cell

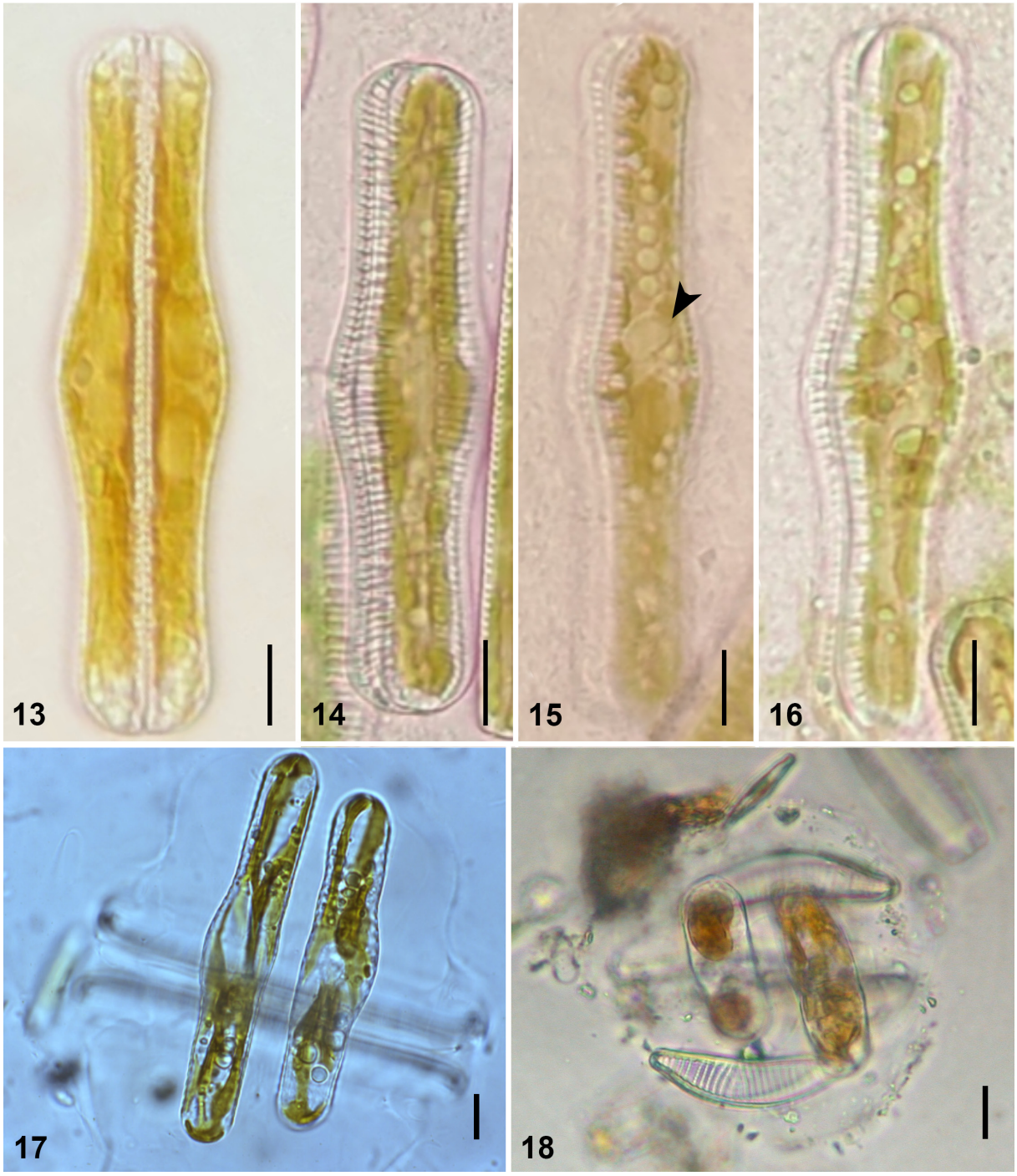


Figs 1–12. *Epithemia* species: (1–11) *E. adnata*; (1) cells with a single highly lobed chloroplast lying along the cell's ventral surface and multiple endosymbiotic cyanobacterial spheroid bodies (arrows); (2–3) normal mitotic cell division producing sibling cells; (4–8) representative cells undergoing internal valve formation by Geitler's (1980) Type 3 where the diminutive cell cut off from the internal cell has no visible cellular contents; a heavily silicified rounded internal valve is formed following an acytokinetic mitosis; (5) cells generated with internal valves, sometimes released from the parent cell; (9) a chain of *Epithemia* cells with the left most cell undergoing Type 3 internal valve formation; with spheroid bodies remaining in the internal cell (arrow); (10) paired internal cells produced within a short *Epithemia* colony and released from the parent cells; the rightmost internal cell has gone through two acytokinetic mitoses during internal valve formation leaving an old internal valve connected to the original parent valve (arrow); (11) a short chain of *Epithemia adnata* cells with multiple examples of Type 3 internal valve formation, including an example of multiple internal or supernumerary valves (arrow); (12) a culture of *Epithemia catenata* produced an internal valve by Geitler's (1980) Type 3 (top cell); no cellular contents remain in the diminutive cell following the acytokinetic mitosis. Scale bars 10 µm.

becomes pycnotic and degrades and was not visible in unfixed and unstained live material.

Epithemia adnata followed internal valve formation by Type 3 of GEITLER (1980) and the cytological sequences described in detail by THALER (1972) for *E. zebra* v. *saxonica*. Several specimens of *Epithemia adnata* showed evidence of multiple acytokinetic mitoses of Type 3, where multiple internal or supernumerary valves were produced (Figs 10–11, arrows).

Cultures of the recently described marine *Epithemia catenata* (SCHVARCZ et al. 2022) showed rare instances of internal valve formation. *Epithemia catenata*



Figs 13–18. *Epithemia* (*Rhopalodia*) *gibba* and *Epithemia turgida*: normal cell division, chloroplast structure, and cyanobacterial endosymbionts (spheroid bodies) in *Epithemia* (*Rhopalodia*) *gibba*; (14–16) internal valve formation in *Epithemia* (*Rhopalodia*) *gibba* following GEITLER's (1980) Type 3, where no cellular contents remain in the diminutive cell cut off following an acytokinetic mitosis, spheroid bodies remain in the internal cell (15, arrow) and a heavily silicified rounded internal valve forms; (17–18) *Epithemia* (*Rhopalodia*) *gibba* (17), Silver Lake Fen, Iowa, USA and *Epithemia turgida* (18), West Lake Okoboji, Iowa, USA undergoing GEITLER's (1973) Type IB1b sexual reproduction with gametangial cells aligned parallel and producing two auxospores expanding perpendicular to the parent cells within a common copulation mucilage. Scale bars 10 μm.

similarly underwent Geitler's Type 3 internal valve formation, creating a small vestigial cell with no cellular content and a more strongly arched single internal valve with no evidence of supernumerary internal valves (Fig. 12).

Cultures of *Epithemia* (*Rhopalodia*) *gibba* showed examples of internal valve production (Figs 14–16) following Geitler's (1980) Type 3, but showed no examples of multiple acytokinetic mitoses or supernumerary valves during internal valve production. The cyanobacterial endosymbionts remained within the internal cells of both *Epithemia adnata* (Fig. 9, arrow) and *Epithemia* (*Rhopalodia*) *gibba* (Fig. 15, arrow).

Examples of internal and supernumerary valve formation were also noted from natural collections. *Epithemia turgida* (Figs 19, 20) and *E. adnata* (Figs 21, 22) from Fishtrap Lake, Minnesota (USA), produced internal valves following Geitler's (1980) Type 3, resulting in a vestigial cell and a single internal valve that showed modified silicification, especially in *E. adnata* (Fig. 22).

In *Epithemia reicheltii* from a natural epilithic collection from 4 m water depth in Lake Superior near Grand Marais, Minnesota (USA), evidence also points to Geitler's (1980) Type 3 internal valve formation, followed by additional acytokinetic mitoses to produce multiple supernumerary internal valves (Fig. 23). The final internal valve produced has a very different outline compared to regular vegetative valves of *E. reicheltii* (Fig. 23).

Last, a collection from Lake Hövsgöl, Mongolia, showed abundant internal valve formation in *Epithemia argus* (Figs 24–27). From cleaned material, this taxon showed evidence of Geitler's (1980) Type 3 formation sequence, but with no indication of multiple acytokinetic mitoses or supernumerary valves.

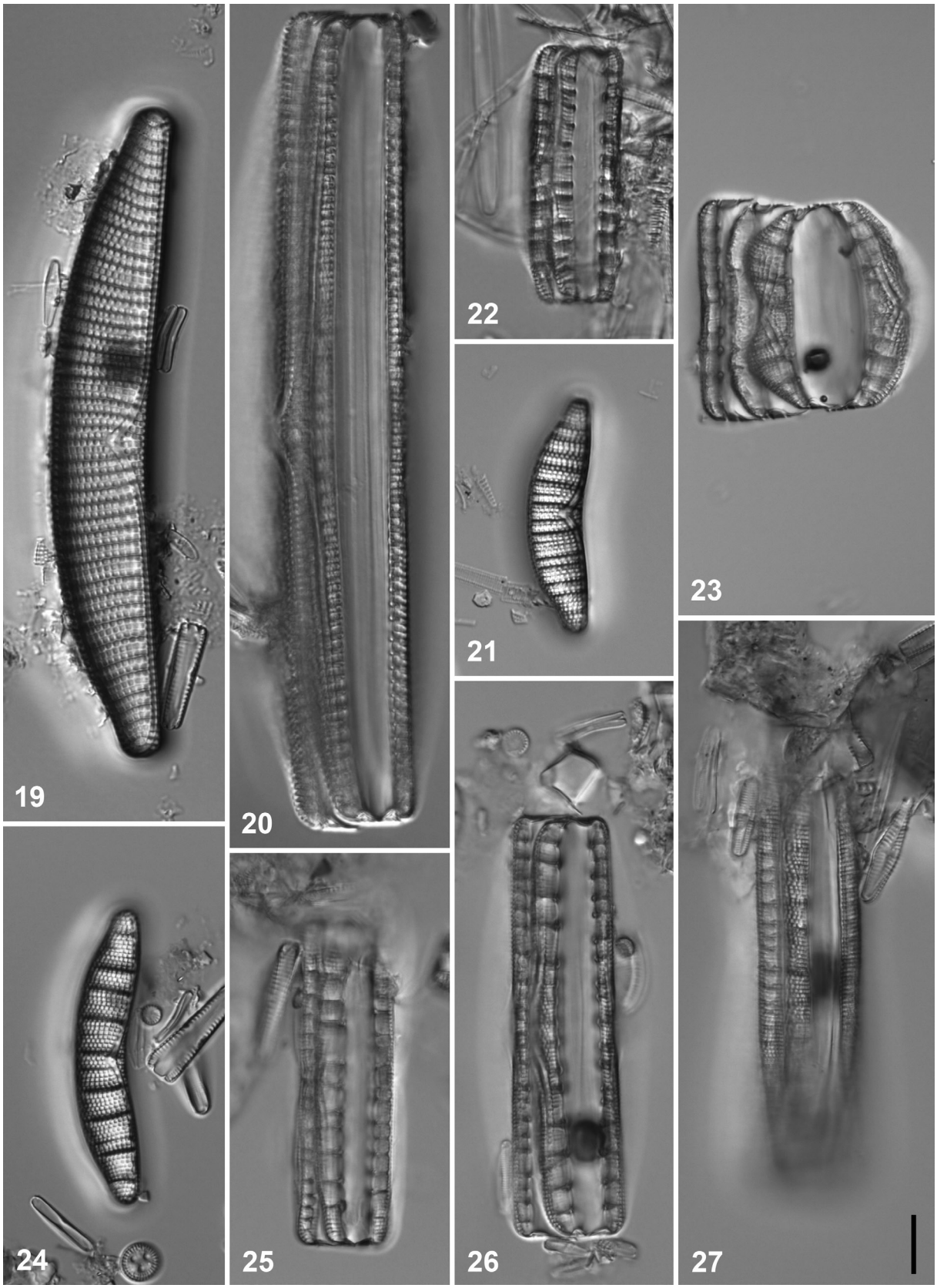
Discussion

With these new data on internal valve formation of Geitler's (1980) Type 3 in *Epithemia adnata*, *E. reicheltii*, *E. turgida, E. argus, E. catenata,* and *Epithemia* (*Rhopalodia*) *gibba*, combined with earlier observations of Type 3 formation by Krasske (1927) and Liebisch (1929) on internal valves in *Rhopalodia musculus*, Müller (1899) on *Rhopalodia gibberula* var. *sphaerula*, Sims (1983) on *E. argus, E. reticulata, and E. hyndmannii*, Kociolek et al. (2024c, 2025b) on *E. alpestris* and *E. schoemanii*, and Thaler (1972) on *E. zebra* v. *saxonica*, two paraphyletic groups of *Rhopalodia* (*Rhopalodia gibba* –group and *R. musculus/gibberula*–group sensu Kociolek et al. 2024b) and one group of taxa traditionally treated as the freshwater *Epithemia* (see phylogenies of Ruck et al. 2016b; Schvarcz et al. 2022; Kociolek et al. 2024c) show homologous patterns of internal valve construction. The paraphyletic group identified by Schvarcz et al. (2022) and Moulin et al. (2024) containing *Epithemia catenata* and two provisionally identified *Rhopalodia* (sp. 13vi08 2B GCCT21 and sp. 3825 12) also represents the "*Rhopalodia musculus/gibberula*–group". In this group, we report Type 3 internal valve construction in *E. catenata*. Importantly, the prevalence of internal valve formation within these Rhopalodiales groups stands in contrast to the infrequency of internal valve formation within most diatom groups and genera.

We also report several examples of supernumerary internal valves as part of Type 3 internal valve production. Some cultured specimens of *Epithemia adnata* showed multiple or supernumerary internal valves (Figs 10–11). In contrast, natural collections of *E. adnata* did not show any evidence of supernumerary internal valves (see Fig. 22). Similarly, natural populations of *E. reicheltii* showed evidence of supernumerary internal valves (Fig. 23). Both of these examples represent the monophyletic group of taxa traditionally treated as the freshwater *Epithemia* (Ruck et al. 2016b). Other examples of supernumerary internal valves have been noted in *Rhopalodia musculus* Kütz. (Krasske 1927; Liebisch 1929), a member of the (*Rhopalodia*) "*Gibberula*–group" (sensu Kociolek et al. 2024b), which also contains *E. catenata*. It is notable that we did not see supernumerary internal valves in our cultures of *E. catenata*. In short, the presence of supernumerary internal valves appears to be a probable modification of Type 3 internal valve formation that is not specific or a characteristic of all individuals within a taxon, can be found in multiple paraphyletic lineages within the Rhopalodiales, and may be a cytological sequence of internal valve formation in cells that continue to experience osmotic stress, as noted by Geitler (1927).

A final paraphyletic group of *Epithemia*/*Rhopalodia* taxa that was resolved by Ruck et al. (2016b), Schvarcz et al. (2022), and Moulin et al. (2024) broadly circumscribes several marine to brackish Rhopalodiales. Detailed multigene phylogenies identify this paraphyletic group variously containing *Rhopalodia operculata* (C.Agardh) Håk./*R. iriomotensis* H.Kobayasi, T.Nagumo et S.Tanaka (Ruck et al. 2016b), *E. pelagica* Schvarcz, Stancheva et Steward/*R. iriomotensis* (Schvarcz et al. 2022), or *E. pelagica*/*R. iriomotensis/Epithemia clementina* prov. nom. (Moulin et al. 2024). At present, there have been no observations on internal valve formation reported within this clade (Ruck et al. 2016b; Schvarcz et al. 2022; Moulin et al. 2024).

Although not yet treated using molecular phylogenetics, Hamsher et al. (2014) considered morphological characters across a wide range of Rhopalodiales. The genus *Tetralunata* and *Epithemia zebra* var. *denticuloides* Hust. were paraphyletic branches within a larger group of Rhopalodiales that also included *Epithemia* sensu lato and *Rhopalodia* sensu lato branches. Within their tree, *Tetralunata* has also been suggested to form internal valves by Geitler's (1980) Type 3. This was recently reported in *T. schoemanii* as *Häutungen* by Kociolek et al. (2024a).



Figs 19–27. *Epithemia* species showing internal valve formation, cleaned and mounted, LM: (19–22) Fishtrap Lake, Minnesota, USA (SMM–MBE1285a); (19) *Epithemia turgida*, vegetative valve; (20) *Epithemia turgida,* Type 3 internal valve formation; (21) *Epithemia adnata,* vegetative valve; (22) *Epithemia adnata,* Type 3 internal valve formation; (23) *Epithemia reicheltii* (Lake Superior, Minnesota) (SMM–MBE2650a), Type 3 internal valve formation followed by additional acytokinetic mitoses to produce multiple supernumerary internal valves; (24–27) *Epithemia argus*, Lake Hovsgol, Mongolia, (SMM–M87); (24) vegetative valve; (25–27) multiple examples of Type 3 internal valve formation. Scale bar 10 μm.

Other cellular and life history traits lend further support to the Rhopalodiales and may inform higher–level classification in the Rhopalodiales with continued and needed observation. First, cells in the broader *Epithemia* and *Rhopalodia* groups all have a single, large, highly lobed and plate–like chloroplast that lies against the cell's ventral surface (Cox 1996; Kamakura et al. 2021; Schvarcz et al. 2022; Moulin et al. 2024). Second, auxosporulation within the subgenera *Epithemia* and *Rhopalodia* (plus *Denticula* Kütz., but see differences between *D. vanheurckii* (=*Tetralunata vanheurckii* (Brun) Hamsher et al.) and *D. tenuis* Kütz.; Geitler 1977) follows Geitler's (1932; 1973) Type IB1b, where gametangial cells align parallel within a copulation mucilage, each gametangium produces two gametes that rearrange incompletely, and the two resulting auxospores align and expand perpendicular to the gametangial valves (Figs 17–18; also e.g., Geitler 1973, 1977; Edlund & Stoermer 1997; Kamakura et al. 2021). Fewer observations on auxosporulation exist among other taxa within the Rhopalodiales (Davidovich & Davidovich 2022; Mann & Edlund 2024); however, the phylogenetic value of auxosporulation within this group has been emphasized (Kociolek et al. 2025a) and represents an area worthy of study. Third, the Rhopalodiales are also well–known for their cyanobacterial endosymbionts (Figs 1, 13; arrows) (spheroid bodies or *Sphäroidkörper*, Geitler 1977; DeYoe et al. 1992; Nakayama et al. 2011; Abresch et al. 2021, 2024; Kamakura et al. 2021; Schvarcz et al. 2022).

We note that spheroid bodies are also reported in some *Denticula*; e.g., *D. vanheurckii* Brun has spheroid bodies, but *D. tenuis* does not (Geitler 1977). *Denticula* is a canal raphe–bearing genus that has been variously considered in the Rhopalodiales or Bacillariales (Mann et al. 2021; Kociolek et al. 2024a). Kociolek et al. (2024a) recently reanalyzed *D. vanheurckii* and transferred it as *Tetralunata vanheurkii*, a group well nested within the Rhopalodiales (Hamsher et al. 2014), but to date untested using molecular systematics. Recent molecular analyses including *D. kuetzingii* Grunow place this *Denticula* taxon well nested in the Bacillariales (Mann et al. 2021) alongside several *Nitzschia*; the broader genus remains a subject for revision (Hamsher et al. 2014; Liu et al. 2017; Kociolek et al. 2024a).

Ultrastructural details of valve and cingulum provide further character support within the Rhopalodiales, but are beyond the scope of this manuscript. Readers are referred to detailed work by Ruck & Kociolek (2004), Ruck et al. (2016b), and Kociolek et al. (2024b, 2025a) among others. Together with Type 3 internal valve production, many lines of evidence of homologous cellular (cell organization and spheroid bodies), cell development (canal–raphe bearing and spheroid bodies), and sexual reproductive (Type IB1b) characters lend support to discerning higher–level relations within the Rhopalodiales as recognized with recent molecular systematic treatments (Ruck et al. 2016a,b; Schvarcz et al. 2022; Moulin et al. 2024).

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