3 Convergent Evolution of Animal-Like Organelles across the Tree of Eukaryotes

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3.1 INTRODUCTION

Morphogenesis through cell-type specification has allowed for incredible anatomical complexity to arise among macroorganisms such as plants, animals, fungi, seaweeds, and kelps, despite these groups evolving multicellularity independently (Parfrey and Lahr, 2013). Much of the field of "evo-devo"—and the crux of this book—is devoted to understanding how distinct cell types evolved and are coordinated to form tissues and organs with sophisticated divisions of labor. In the history of life, this capability is a relatively recent invention, as the last common eukaryotic ancestor was unicellular (as are most eukaryotic species alive today) and necessarily performed all tasks of survival and reproduction within the confines of a single cell membrane. Of course, unicellular eukaryotes or "protists" are still capable of divisions of labor, both spatially (within specialized organelles or cell regions) and temporally (e.g. by changing cell shape over the course of the lifecycle). For instance, protist relatives of animals have multifarious lifecycles that can alternate between solitary and colonial flagellated stages (in choanoflagellates), motile amoebae, and either multicellular or syncytial spheres (e.g., ichthyosporeans), that may encyst or aggregate into multinucleate colonies (e.g., filastereans) (Sebé-Pedrós, Degnan, and Ruiz Trillo, 2017). As with metazoan tissues, each of these stages is associated with distinct gene expression patterns, and uncovering the regulation of these life-stage

transitions may shed light onto the early evolution of cell type specification in animals. Thus, understanding the evolution of complexity in both animals and protists are two intertwined pursuits.

Over the course of eukaryotic evolution, cellular architecture has been expanded by the acquisition of endosymbiotic organelles (e.g., mitochondria, chloroplasts, and their genes) (Gavelis et al., 2015) as well as by the multiplication of endogenous components (e.g., via gene duplications and consequent expansions in protein complexes and pathways) (Mast et al., 2014). While animals form a single eukaryotic clade characterized by embryogenesis (which is generally patterned "top down" by master regulatory genes), protists are paraphyletic—encompassing all eukaryotic diversity excluding animals, plants, and multicellular fungi—and their developmental processes will inevitably be more diverse (Lukes, Leander, and Keeling, 2009). Beyond a handful of well-studied models, the means by which protists establish polarity, symmetry, and the size of their cells and organelles are unclear, and knowledge gaps are particularly glaring for large and/or complex protists (Marshall, 2011). Some lineages have features so ornate and overwrought that they appear very inefficient (e.g. the "kinetoplast" of *Trypanosoma* consists of thousands of interlinked rings of DNA that act as templates for iterative rounds of mitochondrial RNA editing, with no obvious benefit over systems in "normal" cells) (Lukeš, Hashimi, and Zíková, 2005; Lukes, Leander, and Keeling, 2009). Thus, not only are the principles of protist evo-devo unclear, but—as in the field of comparative eukaryotic genomics—it is difficult to determine which cell features result primarily from adaptation, versus developmental constraints, versus "constructive neutral evolution" (wherein genetic drift allows for the accretion of useless details) (Lukes, Leander, and Keeling, 2009; Wideman et al., 2019). In other words, complexity is not necessarily adaptive.

Nevertheless, many elaborate protist features and body plans are adaptive and can perform roles equivalent to what animals accomplish with multitudes of cells and tissue types. For instance, haplozoan dinoflagellates are parasites that look and behave like marine tapeworms (Leander, 2008a). Each organism includes an apical attachment apparatus consisting of a hook and a sucker, microtrich-like surface extensions to facilitate nutrient absorption, and a body composed of linearly-arranged proglottid-like segments that are shed terminally for dispersal along with the of feces the host (a maldinid polychaete or "bamboo worm"). Being only distantly related to animals, this organism represents convergent evolution, where two lineages independently acquire similar structures under similar selective pressures. What is remarkable is that *Haplozoon* is not multicellular—it is a syncytium with a single plasma membrane bounding many nuclei. One nucleus is situated in the "head," while others are arranged serially in segment-like compartments, each of which is delimited by flattened membranous sacs ("alveoli") rather than true cell boundaries (Leander et al., 2002; Rueckert and Leander, 2008; Wakeman et al., 2018). Though the molecular basis of haplozoan development is as yet unstudied, the fact that its cytoplasm is physically partitioned means that morphogenesis could be shaped by differential expression among nuclei. For instance, nuclei in the syncytial alga *Caulerpa* have been found to express certain transport regulators differentially between the leaf-like "fronds" and root-like "stolons" of this meters-long cell (Arimoto et al., 2019).

Unfortunately, it is logistically harder to study how protists achieve differentiation at finer spatial scales.

Protists are capable not only of structural convergence with multicellular body plans, but also of occupying similar niches. For example, vorticellid ciliates greatly resemble entoprocts and some rotifers, all of which are filter feeders that use radially-symmetrical ciliary arrays to capture particles and attach to substrates via an adhesive disk on a long contractile stalk (Rundell and Leander, 2010). Similarly, chonotrich ciliates resemble cycliophorans, both of which have episymbiotic lifestyles near the mouthparts of decapods (e.g., crabs and lobsters), using a ciliary feeding apparatus, stalk, and adhesive disk (Funch and Kristensen, 1995; Obst et al., 2006; Taylor et al., 1995).

The above examples represent structural similarities between the trait complexes of entire organisms, but convergent evolution most often manifests at more specific levels of organization. In the next three examples, we discuss convergent evolution between organs and organelles, using marvelously complex ciliates and dinoflagellates. These are all "non-model" organisms, for they lack sequenced genomes and tools for genetic transformation, and some have not even been cultured (e.g., warnowiid dinoflagellates), but the intricacy of these systems illustrates how much we have yet to learn about protist evo-devo. All three organisms are alveolates, a lineage that diverged from the ancestors of animals over one billion years ago (Parfrey et al., 2011). This distant common ancestry suggests that homology has played little to no role in the canalization of these outcomes (Leander, 2008a,b).

3.2 EYE-LIKE ORGANELLES

Perhaps the most famous example of convergent evolution is the independent occurrence of camera-type eyes: the lens-bearing photosensory structures found in vertebrates and many mollusks, annelids, and arthropods (Gavelis et al., 2017) (Figure 3.1). While each lineage derived its own corneas, lenses, and neural wiring for image formation, these were built on top of a shared toolkit for photoreception that they inherited from their bilaterian common ancestor (i.e., opsins hosted by either rhabdomeric or ciliary cells, which are both specified by the *Pax6* master control gene) (Gehring and Ikeo, 1999). With the possible exception of lensed eyes in cubozoan cnidarians (which are found in the absence of *Pax6* orthologs), camera-type eyes in animals reflect proximate rather than ultimate convergence, as developmental genes played an important role in the canalization of independently derived camera eyes (Leander, 2008a,b).

A case of ultimate convergence with the camera-type eyes of animals exists in the eye-like "ocelloids" of warnowiid dinoflagellates (Leander, 2008b). Ocelloids resemble the camera-type eyes of some animals (e.g., cubozoans) and—when these bizarre cells were first collected from the plankton—were even mistaken for them (Kofoid and Swezy, 1921). The ocelloid consists of a cornea-like layer, a lens, and a pigmented retinal body, albeit at a subcellular scale (Gavelis et al., 2015; Hayakawa et al., 2015) (Figure 3.1). We caution that any role of the ocelloid in phototaxis (movement relative to light) is still speculative, as warnowiids are not yet in culture and

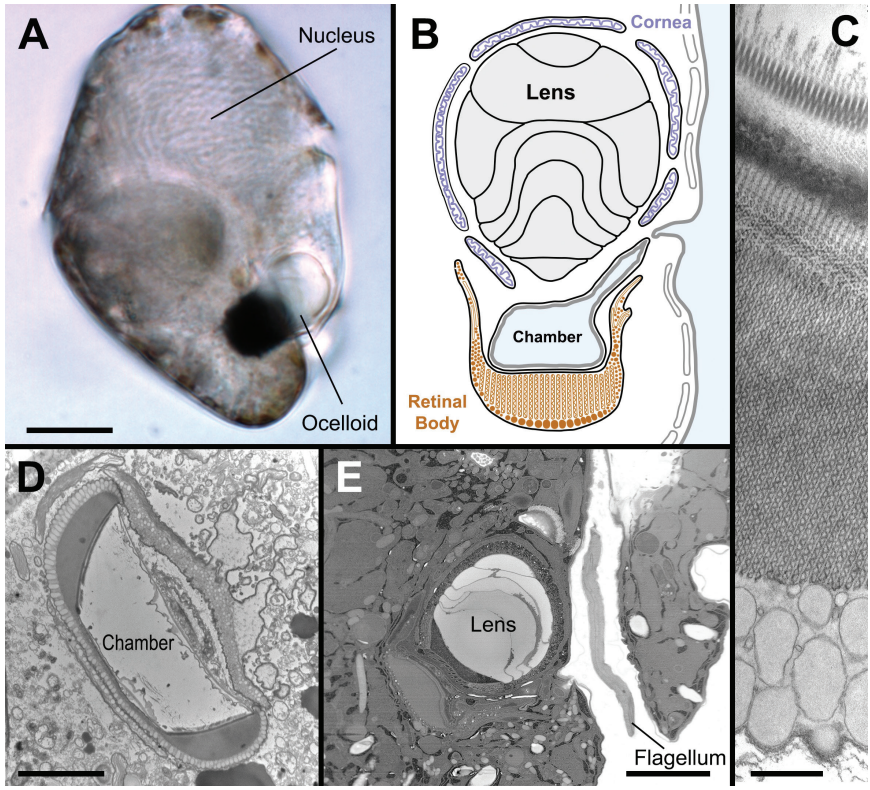

FIGURE 3.1 The eye-like ocelloid of warnowiid dinoflagellates. (**A**) A DIC light micrograph of *Nematodinium*, ocelloid visible in the lower right. (**B**) Diagram of the ocelloid, redrawn from Taylor (1980) with additional details. Red = thylakoid membranes and plastoglobules, purple = mitochondrial cristae, blue = seawater. (**C**) TEM section of the retinal body showing (from bottom to top), plastoglobules, waveform thylakoid membranes, and paracrystalline layers that may somehow serve to process incoming signals. (**D**) An oblique TEM section of the retinal body showing its concave inner surface. (**E**) FIB-SEM section of the ocelloid and longitudinal flagellum. Scale bar = 10 μm in A; 100 nm in C; 2 μm in D, 5 μm in E.

quickly become inactive when isolated from the plankton. Nevertheless, the position and refractive index of the lens suggests that the ocelloid focuses light on the inner surface of its retinal body (Francis, 1967), and while it is conceivable that the ocelloid performs non-sensory functions (e.g., serving a site for photosynthesis), most of its properties are consistent with a phototactic role (e.g. "oculomotor" swiveling motion and its homology to phototactic eyespots in other dinoflagellates, as we will discuss). Our fragmentary knowledge of the ocelloid is still remarkable from an evolutionary and developmental standpoint. Prior to each cell division, the lens, cornea, and retinal body each de-differentiate and divide by binary fission. They are then equally apportioned between daughter cells, each reassembling around an "ocellar fiber" that tethers the ocelloid to a flagellum (Dodge and Greuet, 1987).

It is simplest to describe ocelloid ultrastructure from the outside in: The cornea-like layer is composed of a network of mitochondria that send membranous projections into the surface of the lens (where new crystalline layers are accreted) and may aid in lens formation (Figure 3.1). The lens itself is comprised of concentric vesicles, where the innermost crystals are the most densely packed. At its mid-region, the lens is surrounded by either two or three layers of iris-like rings made of highly reflective crystals (Gavelis et al., 2015; Hayakawa et al., 2015). Together, the lens and rings concentrate light onto the retinal body. The retinal body is separated from the lens by an invagination of the plasma membrane, forming an ocellar chamber filled with "vitreous" seawater. The dark red retinal body is concave and contains densely arranged stacks of waveform membranes that run in parallel to the incoming light (Greuet, 1968, 1977) (Figure 3.1). Despite this unusual arrangement, these membranes were recently shown to be modified thylakoids; in other words, the retinal body is a highly modified plastid (Gavelis et al., 2015). This reveals its homology to simper photoreceptive structures in other dinoflagellates, called "eyespots."

Eyespots are widespread among phototactic protists (Gavelis et al., 2017). They are either cuplike plastids or pigment clusters, sometimes with an overlying reflective layer. Eyespots serve to concentrate light on the base of the flagellum, where photoreceptor proteins are located. Pigmentation shields light from all but one direction—a necessity for directional phototaxis (Jekely, 2009). Unlike eyespots, however, the ocelloid possesses a lens, and it uses this to focus light onto the retinal body, rather than on the flagellum. Since the flagellum is located several microns away from the retinal body (in *Nematodinium*) or on the opposite end of the cell (in *Erythropsidinium*), it is unclear how the ocelloid transmits phototactic signals throughout the cell. In fact, there is still no direct evidence that the ocelloid can detect light at all. While the fragility of warnowiids has precluded behavioral observations, the vast size and high percentage of repetitive and non-coding sequences of dinoflagellate nuclear genomes currently makes warnowiids (and all but the smallest dinoflagellates) intractable for genomic surveys (Lin et al., 2015). A few warnowiid EST sequences are available, but the only sequenced photoreceptor protein is a xanthorhodopsin—a light-driven pump rather than a sensory-type rhodopsin (Hayakawa et al., 2015).

Nevertheless, work on wild-caught cells is allowing certain longstanding questions to be answered, such as the demonstration that the retinal body is a plastid. Moreover, *Erythropsidinium*—the genus with the largest ocelloid—has been reported to possess a striated fiber that could control ocelloid movement. While no evidence for this was provided at the time of description (Dodge and Greuet, 1987), new videographic data has confirmed that the ocelloid can indeed swivel 45°, suggesting that some cytoskeletal element functions akin to an oculomotor muscle (Gómez, 2017). Still, the question of the ocelloid's sensory capabilities remains unknown, as do basic details of warnowiid diversity, ecology, genetics, and behavior.

3.3 STATOCYST-LIKE ORGANELLES

As with photoreception, georeception (syn. gravireception)—the sensing of gravity—has led to the evolution of complex sensory structures across the tree of animals. The

most widespread type of georeceptive organ is called a "statocyst," which consists of a fluid-filled spherical chamber. In its center is a dense mineralized "statolith" that can shift freely as the organism changes position relative to the gravitational field, thereby stimulating mechanoreceptor cells that line the chamber. Among animals, there are many variations on this theme, including statocysts lined by either multi-ciliated or uni-ciliated cells; statoliths that are either free floating or tethered to the statocyst wall; and statolith crystals made of either CaF_2, $CaSO_4$, $CaMgO_4P^+$, or $CaCO_3$ (Ariani et al., 1983; Wiederhold et al., 1989; Becker et al., 2005). Despite this variability, statoliths in some distant lineages of animals (e.g., cnidarians and snails) may be shaped by homologous genes, such as *Pax 2/5/8* and *POU-IV,* though statoliths in ctenophores exist in the apparent absence of *Pax* homologs (Kozmik et al., 2003; O'Brien and Degnan, 2003).

Animal statocysts generally consist of a dozen or more cells, but a case of sub-cellular convergent evolution has manifested in loxodid ciliates (Figure 3.2). These ciliates contain a spherical balancing organelle called the "Müller's vesicle," which contains a mineralized "Müller's body". The vacuole is about 7 μm in diameter and surrounded by flattened vesicles. The Müller's body is about 3 μm in diameter, bound by a double-membrane, and contains ~100 crystals of either $BaSO_4$ (in *Loxodes*) or $SrSO_4$ (in *Remanella*). This crystalized inclusion is suspended from the roof of the vesicle by a sheet of nine microtubules that connects to a modified cilium (immotile, but with a normal array of microtubules). In response to changes in the cell's position, the Müller's body's stalk can swing 90° as if on a hinge, articulating where the microtubules bind to a kinetosome (Fenchel and Finlay, 1986).

Depending on the species of loxodid ciliate, a cell can contain from one to thirty Müller's vesicles, often in different stages of morphogenesis. Development initiates with (1) formation of the crystalized Müller's body at the endoplasmic reticulum, followed by (2) migration of this body to an immotile cilium where (3) a vesicle envelops the crystalized body and cilium, (4) forming a functional Müller's vesicle containing the Müller's body connected by an internal ciliated stalk. Despite its sub-cellular origin, the Müller's vesicle has a similar overall structure and function to the statocysts in animals; changes in the position of the Müller's body prompt the ciliate to briefly "tumble" before altering course (Fenchel and Finlay, 1986). Moreover, laser ablation of the Müller's vesicles in *Loxodes* removed its geotactic behavior (Hemmersbach et al., 1998).

To our knowledge, no statocyst-like structures are found in other protists, even though many other lineages are geotactic. For instance, in *Euglena*, the entire cell is involved in georeception. Because the cell is denser than the surrounding freshwater environment, the weight of its cytoplasm creates tension on the underlying plasma membrane. Stretch-activated channels create a transmembrane calcium influx, informing which side of the cell is facing downward, even as it spins along its axis during helical swimming (Häder et al., 2017). Geotaxis can be disoriented in *Euglena* by placing cells in a medium with equal density, thereby relieving pressure on their mechanoreceptors. This treatment also disrupts geotaxis in *Paramecium* (which lacks Müller's vesicles), but it fails to disorient *Loxodes*, suggesting that its presently uncharacterized georeceptors are internal (Hemmersbach and Häder, 1999). Microgravity experiments

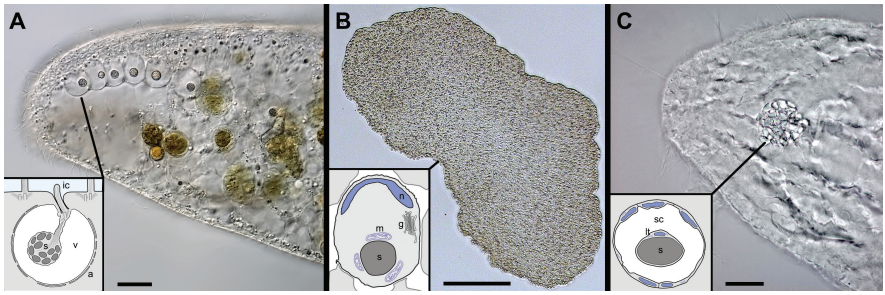

FIGURE 3.2 Statocysts and Müller's vesicle. (**A**) DIC light micrograph of the front of the ciliate *Loxodes magnus*, adorned by at least seven Müller's vesicles. Scale bar = 10 μm. **Inset**. Diagram of the Müller's vesicle, which consists of a statolith (s) suspected from an immotile cilium (ic), and is enclosed by a vacuole (v) lined by flattened vesicles called alveoli (a) (redrawn from Fenchel and Finlay, 1986). (**B**) Light micrograph of *Trichoplax adhaerens*, a deep-branching animal with a flat, nondescript body that lacks an antero-posterior axis but is fringed with up to 150 "crystal cells" around its perimeter (individual crystals not visible at this magnification). Scale bar = 100 μm. **Inset**. Diagram of a crystal cell in *Trichoplax*, showing that its Golgi apparatus (g) and cup-like nucleus (n) reside at the edge of the cell, while the central cytoplasm is occupied by a putative statocyst surrounded by mitochondria (m). (**C**) DIC light micrograph of an unidentified flatworm, in which the spherical crystal statolith has been shattered by pressure on the coverslip. Scale bar = 10 μm. **Inset**. Diagram of the statocyst of *Invenusta paracnida* (a rhabditophoran platyhelminth), in which the statocyst (s) is secreted by – and resides within – a lithocyte (lt) which can tumble freely within a statocyst chamber (sc) lined by sensory epithelium (Redrawn from Ehlers, 1991). Image A modified with permission from Michael Plewka (www.plingfactory.de). Image C modified with permission from Oliver Voigt.

have found georeception to be more sensitive in *Loxodes* than *Paramecium*, with detection limits of 0.16 g versus 0.3 g (Hemmersbach et al., 1996).

It is intriguing that loxodids have evolved statocyst-like organelles for geotaxis while other ciliates have not. This may have been selected for by the fine geotactic balance required by their environment and physiology. Loxodids dwell in marine sediments and stratified lakes and have low oxygen tolerances that restrict them to a narrow part of the oxycline. Even as the oxycline shifts due to tidal cycles or turbulence, loxodids maintain an optimal position via geotaxis, by swimming upward when O_2 levels are too low and downward when they are too high (Fenchel and Finlay, 1984). Given this acuity, why have no similar organelles been found among the myriad of other protists that navigate vertical gradients? Ciliates are larger than most other motile protists, and Fenchel and Finlay (1986) reasoned that the usefulness of statocyst-like vesicles in smaller organisms would be limited by size (Fenchel and Finlay, 1986). Even at a diameter of ~3 μm, (which is large by protist organelle standards) the Müller's body is visibly perturbed by Brownian motion, presumably contributing to sensory background noise. Given that smaller and less dense objects are more easily displaced, Fenchel and Finlay estimated that Müller's vesicles approached the lowest attainable size limits for functional statocyst-type organelles.

Surprisingly, this lower size limit was recently challenged by an animal, as evidenced by the ~2 μm geotactic crystals in *Trichoplax adhaerens*. As an "oligocellular" organism comprised of only six cell types, *Trichoplax* is the simplest free-living animal, with a flat, bilayer body fringed by "crystal cells" of previously unknown function. Each crystal cell is roughly spherical, bearing a central mass of rhomboid $CaCO_3$ crystals surrounded by mitochondria. A recent behavioral analysis of *Trichoplax* found these bodies to tumble freely within the cells, similar to statoliths (Mayorova et al., 2018). Their freedom of movement is facilitated by surrounding cytoplasm that is largely empty of other structures; endomembranes and a cup-like nucleus are displaced to the edge of the cell. Comparison of geotaxis by normal individuals (bearing up to 150 crystal cells) to that of rare individuals lacking crystal cells, found that only the former were able to regulate their depth under experimental conditions (Mayorova et al., 2018). In sum, statocysts seem to have manifested at three levels of organization across eukaryotes: subcellular (as in *Loxodes*), unicellular (as in *Trichoplax*), and multicellular (as in animals with statocysts)—with the first two lineages lacking nervous systems entirely. It remains to be seen whether the "statocyst" of *Trichoplax* is patterned by the same master regulatory genes as other animals. Interestingly, there is preliminary evidence that a *Pax 2/5/8* ortholog (*PaxB*) is expressed in the periphery of *Trichoplax*, where crystal cells are also found (Hadrys et al., 2005).

3.4 NEMATOCYST-LIKE ORGANELLES

Nematocysts (syn. "cnidocysts") are organelles responsible for the infamous stings of jellies, siphonophores, and other members of the Cnidaria and lend the group its name. Concentrated around the tentacles of the polyp or medusa, nematocysts function in prey capture and in defense (Stachowicz and Lindquist, 2000; Bullard and Hay, 2002). Each nematocyst acts as a "single-shot" ballistic weapon at the subcellular level. In one of the fastest mechanisms in biology, a capsule rapidly extrudes a hollow tubule—often in less than a microsecond—thereby puncturing and envenomating prey (Figure 3.3) (Holstein and Tardent, 1984; Nüchter et al., 2006). Discharge is driven by osmotic pressure up to 2,175 psi on the capsule wall, caused by an enrichment of poly-gamma-glutamate within the capsule lumen (Weber, 1989; Özbek et al., 2009). The capsule wall is comprised chiefly of minicollagens, cysteine-rich proteins with extensive disulfide-linkages that make it both tough and elastic (Pokidysheva et al., 2004; David et al., 2008). Upon discharge, the hollow tubule (nested within the capsule) turns inside out and uncoils. This rapid extension provides the force necessary to puncture prey, allowing the everted tubule to deliver its coating of paralytic toxins (Özbek et al., 2009). Most nematocysts are capped by a hatch-like operculum, and the presence/absence of other features, such as stylets and spines lining the tubule (Figure 3.3), have been used to classify nematocysts into several morphotypes, each specialized for certain prey items (Reft and Daly, 2012).

Nematocysts in cnidarians are but one type of projectile organelle, which exist in myriad forms across eukaryotes. Such organelles can be more broadly categorized as "extrusomes," membrane-bound compartments that discharge their contents outside

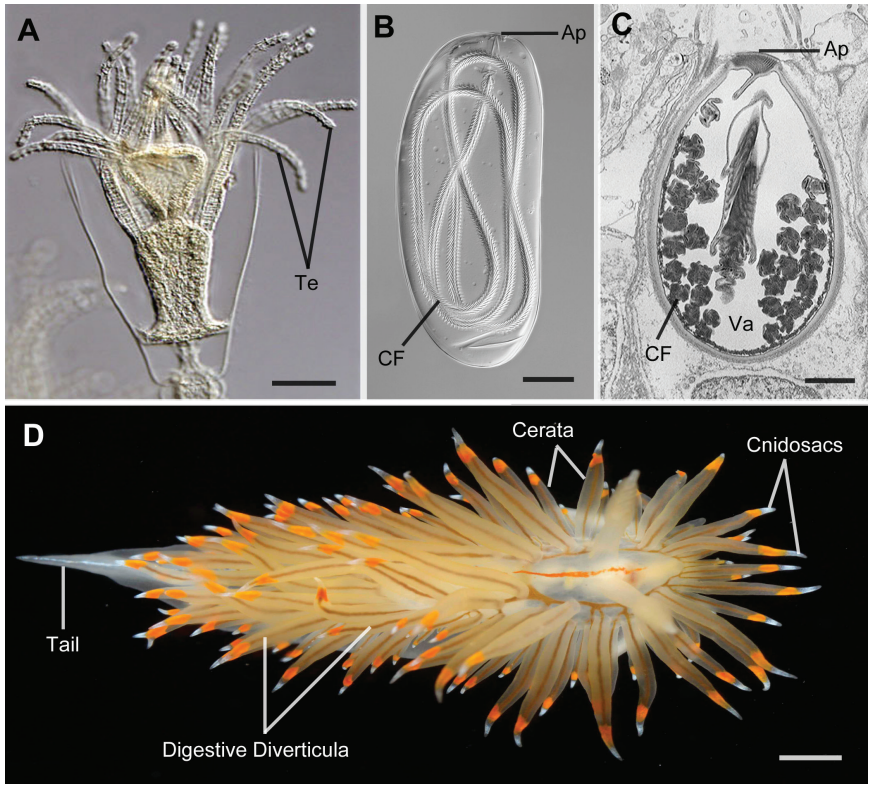

FIGURE 3.3 The position and structure of cnidarian nematocysts and their presence in some sea slugs via kleptocnidae (syn. nematoklepty). (**A**) Light micrograph of a gastrozooid in the hydroid *Obelia* showing the feeding tentacles equipped with cnidarian nematocysts within epidermal cells. (**B**) Light micrograph of a relatively large undischarged nematocyst from the moon jelly *Aurelia* showing the coiled filament that rapidly everts through the apical end of the capsule when stimulated. (**C**) Transmission electron micrograph through a cnidarian nematocyst showing multiple profiles through the coiled filament, the pressurized capsule chamber, and the position of the apical end. (**D**) A photograph of an aeolid nudibranch, *Hermissenda crassicornis*, showing the tentacle-like cerata on the dorsal surface, the position of the posterior end (tail), the digestive diverticula within each ceratum, and the cnidosacs at the tip of each ceratum. The cnidosacs are an extension of the gut lumen (i.e., digestive diverticula) and are filled with undischarged nematocysts stolen from cnidarian prey animals. The collection of (extracellular) nematocysts within the cnidosacs is discharged in a cloud of mucus through a pore when the nudibranch is threatened. Te = tentacles; CF = coiled filament; Ap = apical end; Va = pressurized vacuole. Scale bar = 100 μm in A; scale bar = 1 μm in B and C; scale bar = 0.2 μm in D. Image B is used with permission from W. Nell; image C used with permission from C. Moffet; Image D used with permission from M. LaBarbera.

the cell in response to either chemical or mechanical stimuli (Buonano and Ortenzi, 2018). Extrusome contents range from simple amorphous payloads to highly differentiated forms and can be involved in defense, predation, parasitism, cyst formation, and attachment to substrates (for the most thorough review of exrtrusome ultrastructure, see Hausmann, 1978). Although cnidarian nematocysts are the most intensively studied ballistic organelles, extrusomes are prevalent and diverse among protists, being found in many ciliates, dinoflagellates, euglenozoans, prasinophytes, cryptophytes, chrysophytes, raphidophytes, and chlorarachniophytes (Hausmann, 1978; Kugrens et al., 1994; Rosati and Modeo, 2003), as well as the rare, deep-branching eukaryotes *Ancoracysta* and *Hemimastix*. (Janouškovec et al., 2017; Lax et al., 2018). It is unclear how many times extrusomes evolved, given that their underlying genetics are poorly characterized and because it is difficult to infer homology between one ballistic mechanism and another based on morphology alone. Here, we discuss the "nematocysts" of dinoflagellates, which were the subject of one such homology-oriented debate and are remarkable from the standpoint of convergent evolution.

Characteristic of the dinoflagellate *Polykrikos*, these ~20 μm long extrusomes are only a fraction the size of cnidarian nematocysts but likewise possess a capsule, coiled tubule, stylet, and operculum (Gavelis et al., 2017) (Figure 3.4). Despite being named for their superficial similarity to nematocysts in cnidarians (Butschli, 1873), nematogenesis in *Polykrikos* is unique. Each nematocyst forms in five stacked vesicles that fuse at maturity, whereas cnidarians use a single nematogenic vesicle (Westfall et al., 1983). Furthermore, the tubule forms within the capsule, unlike in cnidarians, where the tubule develops as an outgrowth of the capsule wall, then inverts (Gavelis et al., 2017). Once assembled, the polykrikoid nematocyst migrates to the apical end of the cell and becomes positioned beneath a dense cylindrical organelle, the "taeniocyst," which is not found in cnidarians (Westfall et al., 1983; Hoppenrath et al., 2010) (Figure 3.2). Developmental differences aside, the polykrikoid nematocyst has a similar harpoon-like mechanism, in as far as the stylet is used to puncture prey, allowing the coiled tubule to inject into the prey cell (Chatton, 1914; Kofoid and Swezy, 1921).

This similarity inspired speculation that nematocysts in dinoflagellates and cnidarians are homologous, either via common ancestry or through the dissemination of certain components via horizontal gene transfer (Shostak, 1993; Hwang et al., 2008). However, recent comparative genomic surveys have failed to find evidence of cnidarian-type nematogenic genes (e.g. minicollagens, nematogalectin, or spinalin) in protists and found these genes to be restricted to cnidarians (including their parasitic offshoots, the myxozoans) (Holland et al., 2011; Shpirer et al., 2014; Lin et al., 2015; Gavelis et al., 2017). The only non-metazoan gene that cnidarians are known to employ in nematogenesis is *pgsAA*—which is responsible for biosynthesis of the osmotic propellant poly-gamma-glutamate—and is inferred to have been acquired via horizontal gene transfer from bacteria (Denker et al., 2008). *PgsAA* has not been found in extrusome-bearing protists, where extrusome propellants remain uncharacterized.

Investigations of polykrikoid feeding mechanisms have shown that the specifics of nematocyst function are quite divergent from cnidarians. Microscopic observations

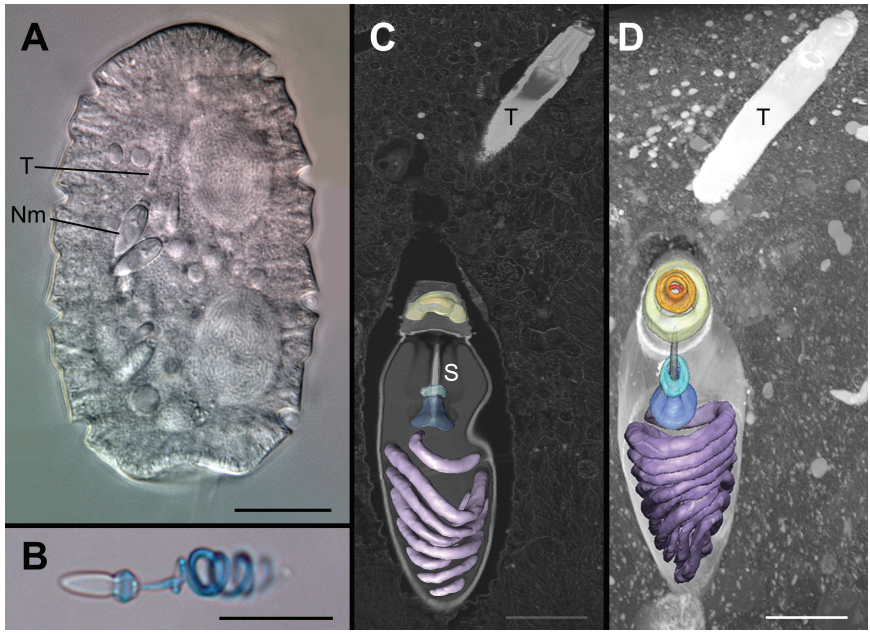

FIGURE 3.4 The structure of "nematocysts" in the dinoflagellate *Polykrikos kofoidii*. (**A**) DIC light micrograph of *P. kofoidii*. Polykrikoids possess two or more nuclei per cell, as well as taeniocysts (T) and nematocysts (Nm). (**B**) A discharged nematocyst in which the tubule and an everted portion of the capsule have been stained with Alcian blue. (**C-D**) Tridimensional reconstructions, based on focused ion beam scanning electron microscopy (FIB-SEM), of a nearly mature nematocyst and taeniocyst that have not yet migrated to the cell periphery. (**C**) Shows that the stylet (S) is poised to pierce the capsule from within. (**D**) Depicts the "operculum" as a complex nozzle formed of three concentric rings (yellow, orange, and red). Upon discharge, the stylet is rapidly ejected through the nozzle, followed by the tubule (purple), which uncoils. The stylet may also function to puncture prey. Scale bar = 20 μm in A & B, 2 μm in C & D.

suggest that the taeniocyst (a dense cylindrical structure positioned distally to the nematocyst in *Polykrikos*) is also an extrusome, as it is osmotically charged and becomes embedded in the prey cell upon contact (Gavelis et al., 2017). Feeding in *Polykrikos* involves two rapid steps: First, the prey contacts the taeniocyst and is adhered, then the nematocyst injects its coiled tubule into the prey cell. The mechanism by which the nematocyst discharges in polykrikoids differs from cnidarians in at least three ways (Gavelis et al., 2017); (1) Prior to exiting the capsule, both the stylet and tubule are launched through a concentric series of three rings, or "nozzle," that appears unique to dinoflagellates. (2) The stylet then punctures the capsule itself, which is entirely enclosed (whereas the capsule in cnidarians possesses a pre-existing opening through which it everts) and is only then able to contact the prey. (3) The tubule is mucilaginous and dissolves in seawater within a minute at room temperature, suggesting that—when injected into prey—it acts as a soluble delivery

system. By contrast, the tubule in cnidarians is formed from insoluble minicollagens proteins, with toxins delivered by an overlying coating. Unfortunately, the contents of the tubule in polykrikoids—or any component of dinoflagellate nematocysts—have yet to be identified.

During this encounter, a motile prey cell may swim away or—if it is another dinoflagellate—may rapidly displace itself by discharging trichocysts. However, as long as the nematocyst is embedded in the prey cell, it remains tethered to *Polykrikos* by a "tow line." (The tow line is not to be confused with the tubule, as it does not originate from within the nematocyst capsule). By an unknown mechanism, *Polykrikos* can retract this tow line into the cytoplasm, drawing in the prey cell to be phagocytosed (Matsuoka et al., 2000). Thus, while the overall act of polykrikoid prey capture can be said to be "harpoonlike"—it includes several unique nematocyst features (a nozzle comprised of concentric rings, a tubule that is mucilaginous rather than insoluble, and a capsule that is completely sealed) and is accompanied by organelles with no clear analogs in cnidarians or other animals (the taeniocyst and the tow line).

As with any case of convergent evolution, the superficial similarities between nematocysts of cnidarians and dinoflagellates probably result from a limited morphospace for biological projectiles. Indeed, the eversible-tube-in-a-pressurized-capsule layout has been put to use in rapidly firing organs/organelles in diverse lineages and at many levels of organization. Multicellular versions of an eversible tube within a capsule are found in the proboscis of nemertean worms, cone snails, and platyhelminths (as the proboscis in kalyptorynchs and as the paracnids in coelogynoporids) and are usually propelled by muscular constriction of a hydrostatic sack (Sopott-Ehlers, 1981; Rundell and Leander, 2014). Single-celled versions of an eversible tube within a capsule are found in microbial eukaryotes like microsporidian fungi and the oomycete *Haptoglossa*, both of which have spores that launch nuclei into the host cytoplasm via an eversible tubule (Glocking and Beakes, 2000) (Figure 3.5). Yet while all predators must capture prey, only a small subset have evolved harpoonlike capture mechanisms. To better understand the selective pressure that drove convergence in these groups, we should consider what ecological commonalities they possess. The most obvious is that these organisms (fungus-like oomycetes, cnidarians, flatworms, and snails) are immotile or slow relative to their prey. Even though *Polykrikos* is a motile planktonic cell, it contends with dinoflagellate prey that have a rapid escape response of their own (trichocysts: spear-like extrusomes that can rapidly polymerize to push the cell several cell-lengths away from its predator). Thus, predatory ballistic structures can pre-emptively immobilize fast prey via adhesion, entanglement, and/or injection of paralytic agents. Pressurized ballistic mechanisms provide not only speed but the force necessary to puncture prey armor, and this feature would be advantageous for intracellular parasites that must penetrate a host (e.g. *Haptoglossa* and microsporidians) (Figure 3.5). By delivering both force and speed without the requirement of advanced neuromuscle systems, ballistic organelles have allowed slow, and/or morphologically streamlined organisms to exploit a broad range of taxa.

While polykrikoids are only known to use nematocysts for predation, cnidarians use nematocysts for both predation and defense, making them unpalatable to most

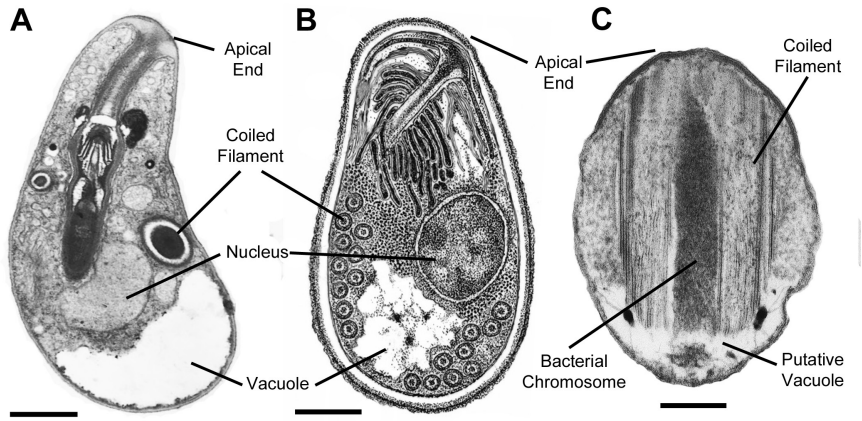

FIGURE 3.5 Convergent evolution of single-cells (in contrast to organelles) capable of discharging a coiled eversible filament within a capsule similar to cnidarian nematocysts. These three examples represent lineages that have diverged from each other over 1 billion years ago. (**A**) A transmission electron micrograph through the gun cell of *Haptoglossa*, a group of parasitic oomycetes (water molds) within the eukaryotic supergroup Stramenopila. (**B**) Schematic illustration of *Potaspora* showing the internal organization of a microsporidian spore, a parasitic relative of fungi within the eukaryotic supergroup Opisthokonta. Haptoglossan gun cells and microsporidan spores share a pressurized vacuole and coiled filament through which the nucleus moves into a host cell following discharge of the filament through the apical end. (**C**) A transmission electron micrograph through an epibiotic verrucomicrobial cell on the surface of the marine euglenozoan *Bihospites*. Like haptoglossans and microsporidians, these complex bacterial cells contain a putative vacuole and a tightly coiled filament that function to discharge a central bacterial chromosome through an apical pore. Scale bar = 0.5 μm in A; scale bar = 0.5 μm in B; scale bar = 0.2 μm in C. Image A modified with permission from Casal et al. (2008); image B modified with permission from Glocking and Beakes (2000); image C first described in Breglia et al. (2010).

non-specialist predators. Nematocysts are so effective at defending these soft-bodied invertebrates that several animal lineages have evolved to "steal" nematocysts for their own defense, with "nematoklepty" estimated to evolve from 9 to 17 times based on phylogenetic inference (Figure 3.3). This has originated most often among platyhelminths but is also found in aeolid nudibranchs and the ctenophore *Haeckelia*—all predators that digest cnidarian tissues and sequester the undischarged nematocysts within epithelial sacs, which they can eject when threatened (Mills and Miller, 1984; Vorobyeva et al., 2017; Krohne, 2018). Multiple lineages of crustaceans have also appropriated nematocysts indirectly through various forms of ectosymbiosis, such as by carrying cnidarians on mollusk shells, growing them directly on their carapaces, or—in the "boxer crab" *Lybia*—by wielding anemones in modified claws that they use both to ward off predators and to cleave the polyps for asexual propagation (Schnytzer et al., 2013, 2017). This diversity of "second-hand" extrusomes in mollusks, arthropods, platyhelminths, ctenophores, and even some protists (e.g., two genera of ciliates and euglenozoans host bacterial episymbionts that violently lyse

upon contact to deter prey Breglia et al., 2010; Petroni et al., 2000; Rosati et al., 1999) (Figure 3.5)—suggests that strong selective pressures exist for the acquisition of ballistic defenses.

Nematogenic machinery, function, and development in cnidarians are highly conserved; all exhibit a basic harpoonlike function, varying only in size and the presence or absence of stylets and opercula. By contrast, not only do protists possess harpoon-like extrusomes, but also ballistic mechanisms involving proteinaceous syringes ("taeniocysts" and "toxicysts"), unfurling ribbons ("ejectisomes"), polymerizing spears ("trichocysts"), and lattices that rapidly expand upon hydration ("mucocysts") (Hausmann, 1978). Several predatory protists host multiple ballistic types simultaneously, with some specialized for predation and others for defense (e.g., a typical cell of *Polykrikos kofoidii* wields half a dozen nematocysts and taeniocysts, hundreds of mucocysts, and ~1,000 trichocysts), whereas cnidarians bear at most one nematocyst per cell. In protists, the molecular means by which extrusome arsenals are organized within the endomembrane system have only been experimentally studied in two ciliates (*Tetrahymena* bearing mucocysts, and *Paramecium* bearing trichocysts), and these studies implicate gene family expansions for CORVET and SNARE proteins, which facilitate vesicle tethering and fusion at membrane interfaces (Sparvoli et al., 2018; Plattner, 2017). It remains to be seen whether these same gene families expanded in dinoflagellates. While trichocyst matrix proteins appear homologous across dinoflagellates and ciliates based on sequence similarity (Rhiel, Wöhlbrand, and Rabus, 2018), proteins from other dinoflagellate extrusome types are as yet uncharacterized.

Even within a single extrusome type (nematocysts), dinoflagellates display greater structural variation than in cnidarians. For instance, not all dinoflagellate nematocysts have a clear harpoon-like arrangement. Nematocysts in warnowiids (aforementioned for their eye-like ocelloids) consist instead of 7 to 14 "barrels" in a radial arrangement reminiscent of a Gatling gun (Gavelis et al., 2017). Warnowiids are closely related to polykrikoids and share distinct features of nematocyst development (both involve multiple vesicles that fuse at maturity and become encased by a striated lattice with identical periodicity). The structural variation between nematocysts in these two groups is remarkable, given their relatively recent common ancestor (dated 120 MYA by molecular estimates), whereas cnidarians are likely to be at least four times as old, and their nematocysts show no deviations from a strict coiled-tubule-in-a-capsule formula (Cunningham et al., 2017; Žerdoner Čalasan, Kretschmann, and Gottschling, 2019). Genetic studies have found that the subtle differences across cnidarian nematocysts can be attributed in part to lineage-specific gene/domain duplications for proteins such as minicollagens (David et al., 2008; Shpirer et al., 2018), as well as the emergence of new protein isoforms (e.g., nematogalectin) via alternative splicing (Hwang et al., 2010). While the processes driving extrusome diversification in dinoflagellates are unknown, our understanding of them can be guided by the approaches used in the study of cnidarian nematogenesis. Given that *Polykrikos kofoidii* can be cultured, next steps should involve: (1) transcriptomics, (2) bioinformatic mining for potential structural proteins (e.g. cysteine-rich and coiled-coil domains are associated with the disulfide linkages and striated protein

layers, which are common in extrusomes), (3) bulk proteomics on nematocysts to verify that candidate proteins are present, and lastly (4) immunolabeling to assess the location (and by inference, the function) of candidate proteins in charged and undischarged nematocysts.

3.5 CONCLUSIONS

Convergent evolution reflects similar selective pressures operating in similar environments. As discussed above, different examples of convergent evolution can occur across fundamentally different levels of biological organization and over vast phylogenetic distances. For instance, multicellular traits in some organisms have converged with either single-celled or subcellular traits in other organisms that have diverged from one another over 1 billion years ago. As in any comparative field, concepts such as "complexity" and "evolutionary lability" are relative. By incorporating protists in macroevolutionary discussions, we are able to consider animal cell features within a much larger morphospace. For instance, it is clear that protists have evolved certain sensory structures (e.g., statocyst-like Müller's vesicle and eye-like ocelloids) in the absence of gene complements that are familiar from animal models (e.g., *PaxB* and *Pax6* master control genes) and that they have formed these structures at fundamentally different levels of organization (organellar versus unicellular versus multicellular in animals). Not only do protists provide examples of extreme subcellular differentiation, they highlight how little we understand about the developmental processes that promote and constrain the evolution of complexity at the cellular scale.

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