Integrative and Comparative Biology

Integrative and Comparative Biology, volume 64, number 5, pp. 1255–1268
https://doi.org/10.1093/icb/icae101

Society for Integrative and Comparative Biology

SYMPOSIUM

Acquisition of Polymorphism in the Chordate Doliolids

C. J. Pickett[*], Joseph Ryan[†] and Bradley Davidson[*,1]

[*]Department of Biology, Swarthmore College, 19081, Swarthmore, USA; [†]Whitney Laboratory for Marine Bioscience, 32080, St. Augustine, USA

From the symposium "Chordate Origins, Evolution and Development" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 16–March 31, 2024.

[1]E-mail: bdavids1@swarthmore.edu

Synopsis In polymorphic organisms, a single genome is deployed to program numerous, morphologically distinct body plans within a colony. This complex life history trait has evolved independently within a limited subset of animal taxa. Reconstructing the underlying genetic, cellular, and developmental changes that drove the emergence of polymorphic colonies represents a promising avenue for exploring diversifying selection and resulting impacts on developmental gene regulatory networks. Doliolids are the only polymorphic chordate, deploying a single genome to program distinct morphs specialized for locomotion, feeding, asexual, or sexual reproduction. In this review, we provide a detailed summary of doliolid anatomy, development, taxonomy, ecology, life history, and the cellular basis for doliolid polymorphism. In order to frame the potential evolutionary and developmental insights that could be gained by studying doliolids, we provide a broader overview of polymorphism. We then discuss how comparative studies of polymorphic cnidarians have begun to illuminate the genetic basis of this unusual and complex life history strategy. We then provide a summary of life history divergence in the chordates, particularly among doliolids and their polymorphic cousins, the salps and pyrosomes.

Overview of polymorphism

Polymorphic colonies are composed of individuals, termed morphs, castes, or zooids, that exhibit an array of distinct body types. The best-known representatives of such colonies are found in the social insects. In these colonies, asexual reproduction produces genetically similar individuals that take on distinctive caste-specific morphologies that are generally induced by environmental inputs (Abouheif 2021). Polymorphic colonies also occur within a limited set of marine invertebrate taxa, including the cnidarians, bryozoans, and tunicates (Harvell 1991; Hiebert et al. 2021). However, marine invertebrate and social insect forms of polymorphism are fundamentally distinct in relation to two criteria. First, marine polymorphic colonies often consist of individual zooids that are physically integrated. This property is clearly illustrated in bryozoans, in which highly distinctive zooids specialized for feeding, defense, or reproduction form branching or sheet-like colonies (Simpson et al. 2017; Schack et al. 2019). Second, these marine colonies are produced through asexual budding and therefore they are isogenic, meaning that a single genome is shared by all of the distinct morphs in a colony. Along with the bryozoans, isogenic polymorphism is frequently observed in a subset of cnidarian taxa, including Hydroida and Siphonophora (Beklemishev et al. 1969; Harvell 1994). Outside of cnidarians and bryozoans, isogenic polymorphism is extremely rare. Remarkably, one of these rare exceptions is found in an invertebrate chordate clade, the doliolids (Gibson and Paffenhöffer 2002; Piette and Lemaire 2015).

Exploration of isogenic polymorphism may provide fundamental insights into developmental genetics and evolution

Investigations into the genetic basis of isogenic polymorphism have the potential to reveal fundamental principles of developmental gene regulatory network

Advance Access publication July 11, 2024

(GRN) structure and function. Mutations that impact the functional output of developmental GRNs are thought to drive trait acquisition, particularly in cases that involve major changes in morphology (Figs. 1A and B) (Shubin et al. 2009; Davidson 2010; Peter and Davidson 2011; Richardson 2022; Cutter 2023). In contrast, the acquisition of isogenic polymorphism must include mutations that generate multiple, alternate functional outputs of developmental GRNs (Fig. 1C), along with mutations that alter GRN architecture, so that these distinct developmental programs can be deployed at the appropriate life history stages or in appropriate regions of the colony (Harvell 1994; Lidgard et al. 2012). Thus, the study of isogenic polymorphism has the potential to address fundamental questions about architectural principles that dictate GRN function, robustness, and versatility. How are polymorphic gene networks structured to ensure robust execution of each developmental program while maintaining the versatility required for execution of multiple programs? Are there specific structural elements of GRNs that are more versatile, allowing them to be deployed in multiple, distinct programs vs. other structural elements that are more constrained, preventing them from contributing to program re-deployment?

Investigations into the acquisition of isogenic polymorphism also have the potential to illuminate principles of diversifying selection. In monomorphic organisms, alterations in developmental programs result in novel phenotypes within the population that are subjected to selection. Intriguingly, in polymorphic organisms multiple, distinct phenotypes (morphs) present in each individual are subjected to selection. Thus, the study of isogenic polymorphism has the potential to shed light on antagonistic selection between morphs (positive selection in regards to one morph is countered by potential negative impacts on the fitness of another morph) (Goedert and Calsbeek 2019). Further studies of isogenic polymorphic organisms will complement related studies of polyphenic social insects (Stern 2000; Linksvayer et al. 2012; Bonasio 2014; Pyenson and Rehan 2024) along with more widespread and better characterized instances of dimorphism, including sexual dimorphism (Williams and Carroll 2009; Herpin and Schartl 2015) or life history dimorphism between larval and adult forms (Aguirre et al. 2014; Truman 2019; Yamakawa et al. 2019). Integration of these efforts will represent a powerful platform for exploring the impact of antagonistic selection on dimorphic or polymorphic traits along with associated impacts on the cellular and genetic mechanisms that produce these traits.

Studies of isogenic polymorphism in the siphonophores

To date, the most progress on understanding polymorphic traits from a morphological and genetic perspective comes from work on siphonophores. Isogenic polymorphism within the siphonophores is strikingly represented by *Physalia physalis*, commonly referred to as the Man-O-War (Munro et al. 2019). These colonial organisms produce seven distinct isogenic zooids through asexual budding. These include a large pneumatophore (the float) specialized for locomotion, gastrozooids and tentacular palpons specialized for feeding and prey capture, respectively, and four additional zooids (gonophore, palpons, nectophores, and jelly polyps) that form a detachable reproductive structure (the gonodendron). However, recent studies have focused on other siphonophore species that are easier to collect and culture (Dunn and Wagner 2006). Intriguingly, the developmental mechanisms generating polymorphic siphonophore colonies are highly diverse (C. Carre 1967; Carre 1969; Carre and Carre 1991; D. Carre 1967; Siebert et al. 2015). Thus, comparative studies within siphonophores have the potential to reveal conserved, potentially ancestral mechanisms driving the emergence of polymorphism in this clade along with elucidating how these mechanisms have diverged. A number of studies have examined micro-anatomical differences between zooids using modern techniques but this approach has only been applied to a limited subset of polymorphic siphonophore species (Mackie 1960; Carre 1969; Bardi and Marques 2007; Church et al. 2015; Siebert et al. 2015). Additionally, some insights have been gained regarding the identity, location, and potency of stem cell lineages that generate different zooids (Siebert et al. 2015). More recent work has leveraged RNA sequencing (RNA-seq) to investigate polymorphism in these lineages. Extensive RNA sequencing of siphonophore transcriptomes has generated a robust understanding of phylogenetic relationships in this clade (Munro et al. 2018). Furthermore, studies of RNA-seq data have begun to illuminate differences in gene expression that may underlie phenotypic differences between zooids, laying the groundwork for deciphering the regulatory mechanisms that dictate zooid-specific expression patterns (Siebert et al. 2011; Plachetzki et al. 2014; Sanders et al. 2014; Macrander et al. 2015; Sanders and Cartwright 2015). Most recently, Munro and colleagues conducted a large-scale comparative RNA-seq analysis incorporating zooid-specific transcriptomes from seven siphonophore species (Munro et al. 2022). Critically, they refined their comparative

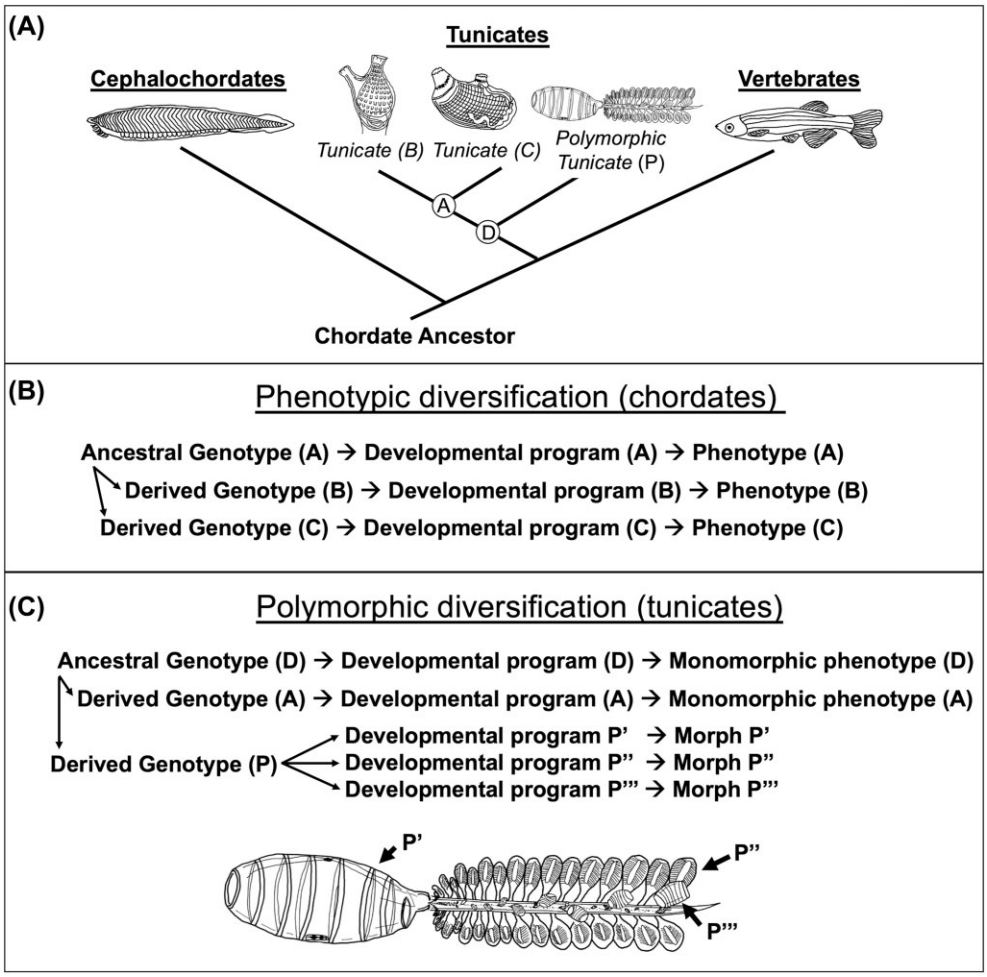

Fig. 1 Acquisition of non-polymorphic traits vs. polymorphic traits. (**A**) Chordate phylogeny. (**B**) Schematic depicting the acquisition of divergent phenotypes in two species (B and **C**) that share a recent common ancestor (A, see text). (C) Top: Schematic depicting the acquisition of polymorphism from a nonpolymorphic ancestor D (also represented in panel A) along with multiple morph-specific phenotypes (P' vs. P'' vs. P'''). On the bottom of this figure panel, a doliolid colony is used to illustrate different morphs.

analysis approach to allow them to normalize expression across these transcriptomes, eliminating potential noise resulting from differences in sequencing depth and overall data quality. They also incorporated a novel approach, species branch filtering, for enhanced identification of orthologous gene sets. These combined approaches allowed them to identify zooid-specific expression patterns in each species and productively compare these patterns across species. Through this analysis, they identified 349 zooid/tissue-associated genes that were enriched for a subset of presumptive functions, including embryonic development and morphogenesis. These genes included orthologs to signaling and transcription factors (such as *WNT3A*, *FGF20*, *SOX21*, and *FOXL1*) that were uniquely and consistently expressed in specific types of zooids across all or most of the species that were examined. They also identified shifts in zooid-associated gene expression patterns that may have contributed to evolutionary divergence of zooid developmental programs.

The deployment of single-cell RNA sequencing (scRNA-seq) promises to provide transformative insights into the evolutionary emergence and diversification of isogenic polymorphism within the siphonophores and other taxa. Recently developed approaches for cross-species, phylogenetically informed comparative analysis (Liang et al. 2015; Musser et al. 2021; Tanay and Sebé-Pedrós 2021; Gilbert et al. 2022; Mah and Dunn 2024) will be essential in using this technique to investigate GRN structure and function in isogenic polymorphic colonies and explore the evolution of this complex and fascinating life history strategy.

Life history divergence in the chordates

The phylum *Chordata* includes the vertebrates and two invertebrate lineages, the cephalochordates and tunicates (Figs. 1A and 2). Cephalochordates and most vertebrates have a monomorphic life history, with larval or juvenile stages that are morphologically similar to the adults. In contrast, tunicates often employ a biphasic (dimorphic) life history strategy in which fertilized eggs develop into distinct free-swimming tadpole-like larvae, which subsequently settle and metamorphosize into sessile filter-feeding adults (Karaiskou et al. 2015). Current phylogenies place the cephalochordates as sister to all other chordates with tunicates sister to the vertebrates (Delsuc et al. 2006). According to current models, tunicate metamorphosis arose secondarily after divergence from the tunicate/vertebrate shared ancestor (Paris and Laudet 2008; Fodor et al. 2021). Thus, tunicate larvae are no longer considered to be homologous to either the larvae or adults of other chordates.

The roughly 5,000 known tunicate species have been traditionally subdivided into three major clades based on morphology and life history: the Ascidiacea, Thaliacea, and Appendicularia (Fig. 2; DeBiasse et al. 2020). Molecular phylogenetic and phylogenomic studies have challenged the validity of this arrangement as Thaliacea, the pelagic group that includes doliolids, is consistently recovered as nested within the benthic clade that contains Ascidiacea (Fig. 2; Swalla et al. 2000; Delsuc et al. 2018).

Ascidians are characterized by pelagic non-feeding tadpole larvae that metamorphosize into sessile, vase-like, filter-feeding adults. The ascidians include both solitary and colonial forms and include two major, phylogenetically distinct taxa, Phlebobranchia and Stolidobranchia. Phylogenomic evidence has recently challenged the monophyly of the solitary Phlebobranchia, which appears to encompass Aplousobranchia, a lineage that is almost entirely colonial (Swalla et al. 2000; Delsuc et al. 2018; DeBiasse et al. 2020). This is particularly important as Phlebobranchia includes *Ciona robusta*, the primary model tunicate species, which has been subjected to extensive characterization, including comprehensive single-cell RNA sequencing of embryonic stages (Cao et al. 2019; Zhang et al. 2020; Fiuza and Lemaire 2021; Bump and Lubeck 2023).

Appendicularians and thaliaceans are exclusively pelagic. The Appendicularians have a body plan that closely resembles the tadpole larvae of other tunicates. Recent studies indicate that Appendicularians arose from a metamorphic, ascidian-like ancestor (Stach et al. 2008; Onuma and Nishida 2022) and have undergone extensive gene loss associated with the loss of the ancestral, biphasic life history (Ferrandez-Roldan et al. 2021; Marti-Solans et al. 2021). Thaliaceans are divided into three orders: the pyrosomes (*Pyrosomatida*), salps (*Salpida*), and doliolids (*Cyclomyaria*), all of which form colonies for part or all of their life cycle (Fig. 3; Godeaux and Harbison 2003; Piette and Lemaire 2015). Salps form chain-like colonies that participate in alternating, semi-independent asexual and sexual generations (Fig. 3A). A single asexually reproductive oozooid buds to produce a long chain of sexually reproductive blastozooids. This chain detaches to form an independent monomorphic colony. Hermaphroditic blastozooids often carry a single embryo, which will be released and develop into an oozooid to reinitiate the cycle. Thus, salps can be considered dimorphic as they have distinct asexual and sexual zooids. Self-fertilization within the colony is facilitated by staggered gametogenesis, younger blastozooids first producing eggs, which are fertilized by sperm produced subsequently as blastozooids mature (Lambert 2005; Piette and Lemaire 2015).

Pyrosomes are sock-like colonies consisting of many physically linked but physiologically independent zooids (Godeaux 1957; Alié et al. 2021; Lilly et al. 2023). Pyrosome colonies are derived from a cyathozooid that produces a chain of four primary buds (primary ascidiozooids) before being resorbed by the forming colony. The primary ascidiozooids produce chains of composite buds that mature into asexual or sexually reproductive secondary ascidiozooids that constitute the bulk of the colony (Fig. 3B) (Godeaux 1957). Thus, pyrosomes can be considered isogenic polymorphs, as a single individual produces three distinct morphs. Secondary ascidiozooids are hermaphroditic and exhibit staggered gametogenesis similar to that observed in salps (Bone 1998). As detailed below, doliolids have a more complex and highly polymorphic life history consisting of four morphologically distinct adult forms (Figs. 3C and 4A) (Barrois 1885; Neumann 1906; Paffenhöfer and Köster 2011).

Although each thaliacean order is characterized by many unique, taxon-specific traits, phylogenetic analyses indicate that Thaliacea is monophyletic (Stach and Turbeville 2002; Tsagkogeorga et al. 2009; Delsuc et al. 2018; Braun et al. 2020; DeBiasse et al. 2020). Phylogenetic analyses also indicate that doliolids are sister to the rest of the Thaliacea (DeBiasse et al. 2020). However, further sequencing and analyses will be required to generate a robust and detailed thaliacean phylogeny.

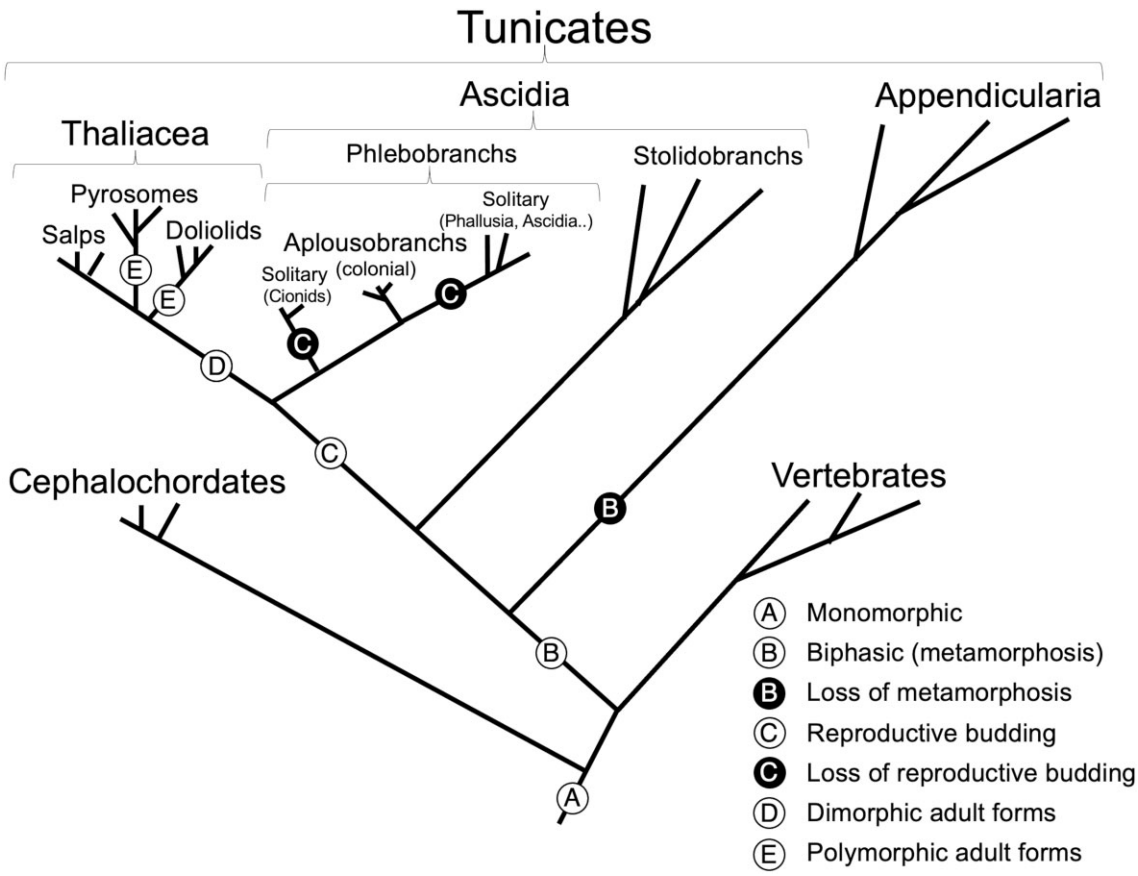

Fig. 2 Model for major life history changes within the chordates. Lettered circles indicate hypothesized major gains or losses of life history traits as labeled on the lower right.

Doliolid taxonomy and anatomy

Doliolids are the only family (*Doliolida*) in the order *Cyclomyaria*, as originally described by Quoy and Gaimard (1834). They are characterized by a barrel-shaped body encircled by a series of muscular hoops (Doliolum: *Latin* barrel) (Fig. 4). The anterior end of this barrel is an incurrent (buccal) siphon and the posterior end is an excurrent (cloacal) siphon. The inside of the barrel is bisected by a branchial septum on which there are a number of ciliated gill slits, similar in morphology and function to the pharyngeal gill slits of other tunicates (as illustrated in the drawings of a phorozooid, gonozooid, and early nurse in Fig. 4A). Ciliary flow generated by these slits creates a feeding current and also propels them slowly forward. Contraction of the circumferential muscles allows them to move rapidly away from aversive stimuli. The most recent classification places doliolids into two suborders: the *Doliolidina* with a regular barrel-like body encircled by 8–9 muscle bands, and the *Doliopsidina* with more globular bodies containing only 5 muscle bands (Godeaux 2003; Godeaux and Harbison 2003). Each of these sub-orders is subdivided into two families (the *Doliolidina* include *Doliolidae* and *Doliopsididae*, while the *Doliopsidina* include *Doliopsidae* and *Paradoliopsidae)*. Excepting the *Doliolidae,* each of these families contains only one genus and three or less species. There are currently ~75 known doliolid species and most of these species are assigned to one of the four genera within the *Doliolidae* (*Dolioloides, Doliolina, Dolioletta*, and *Doliolum*).

Collection and identification of doliolid species is challenging because they are fragile and likely to be damaged when collected from the plankton. Indeed, many of these species (including all of the *Doliopsidina* species*)* are represented by a single life history stage. Additionally, because doliolids are extremely difficult to culture, species are often represented by free-living zooids that cannot be rigorously categorized (Godeaux and Harbison 2003). Molecular sequencing can be used to bypass this issue, but currently the broadest effort only included sequencing of mitochondrial cytochrome oxidase 1 subunit fragments from seven *Doliolidae* species (Garic and Batistic 2022). As predicted by morphological characteristics (Godeaux 1998; Godeaux 2003), molecular phylogenetics supported two *Doliolum* species and two *Dolioletta* species as

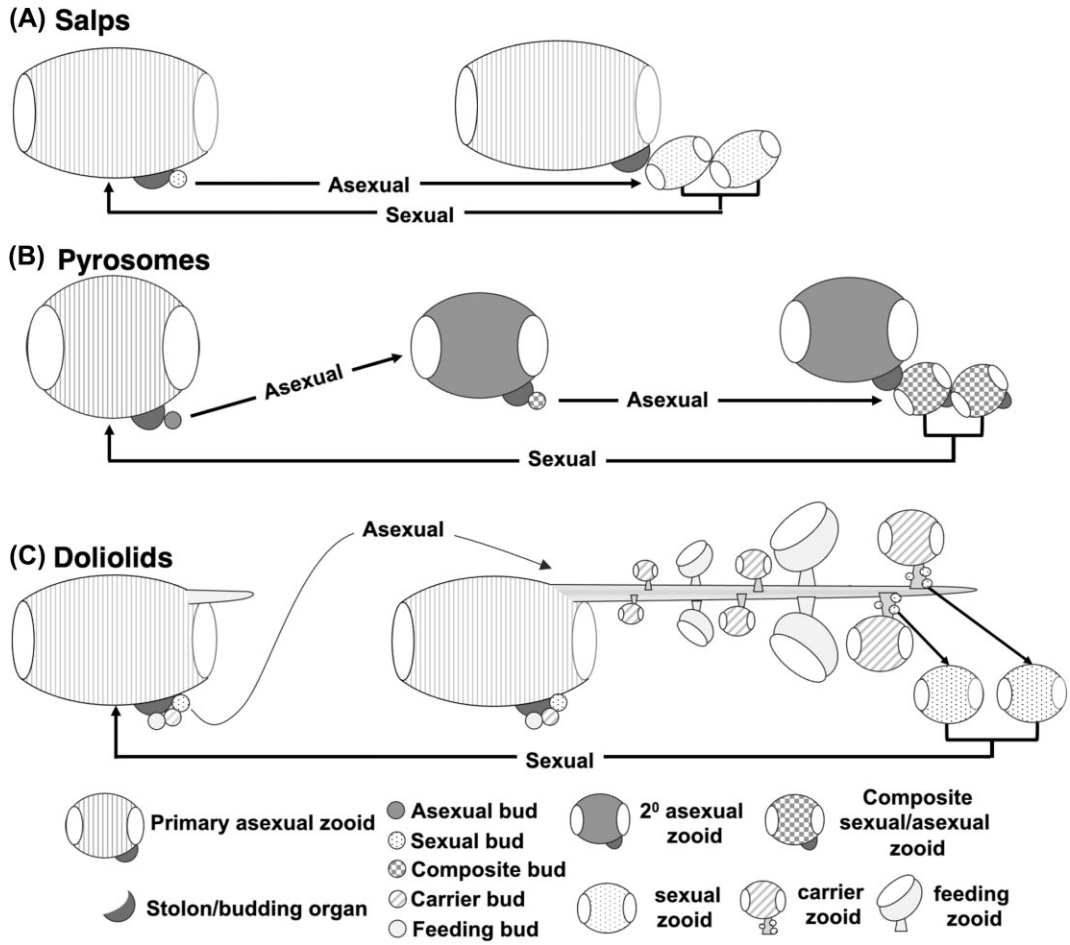

Fig. 3 Clade-specific life-history patterns in the thaliaceans. Simple schematics comparing life history patterns in (**A**) salps, (**B**) pyrosomes, and (**C**) doliolids. Asexual budding or sexual reproduction represented by labeled arrows. Buds and zooid types labeled on the bottom of the figure. See text for details.

monophyletic groups, while two *Doliolina* species were not found to be monophyletic (Fig. 5) (Garic and Batistic 2022). Mitochondrial genomic sequencing was performed for *Doliolum nationalis* (Yokobori et al. 2005), but no nuclear genomes have been published for any doliolid species.

Doliolid ecology

Doliolids inhabit nearly all open-water marine environments (Bone 1998; Deibel and Paffenhöfer 2009). Although doliolids are particularly abundant in continental shelf upwellings or in sub-tropical and tropical regions, some species are found in the Mediterranean, north Pacific, and north Atlantic oceans (Bone 1998; Gibson and Paffenhöfer 2000). While the majority of described species inhabit the euphotic surface waters of the epipelagic zone, several species have been discovered to occupy the aphotic twilight zone (Bone 1998; Deibel and Paffenhöfer 2009). A recently discovered, putatively carnivorous species of doliolid, *Pseudusa bostigrinus*, was found at depths of over 1800 meters (Robison et al. 2005).

Doliolids can undergo planktonic blooming to generate remarkably dense populations. For example, *Dolioletta gegenbauri* sexual zooids (gonozooids) have been recorded at densities of up to 1000 individuals/m^{-1} during favorable bloom conditions (Deibel 1985; Deibel and Lowen 2012; Paffenhöfer 2013). Due to the abundance of doliolids and their pervasive distribution, they make substantial contributions to planktonic food webs and carbon cycling in numerous marine ecosystems (Gibson and Paffenhöfer 2000; Takahashi et al. 2015; Walters et al. 2019b; Frischer et al. 2021).

Doliolid development and larval morphology

Doliolids are also the only thaliacean order in which a presumably ancestral tadpole-like larval stage has been retained. Remarkably, doliolid development remains very poorly characterized. There are only a handful

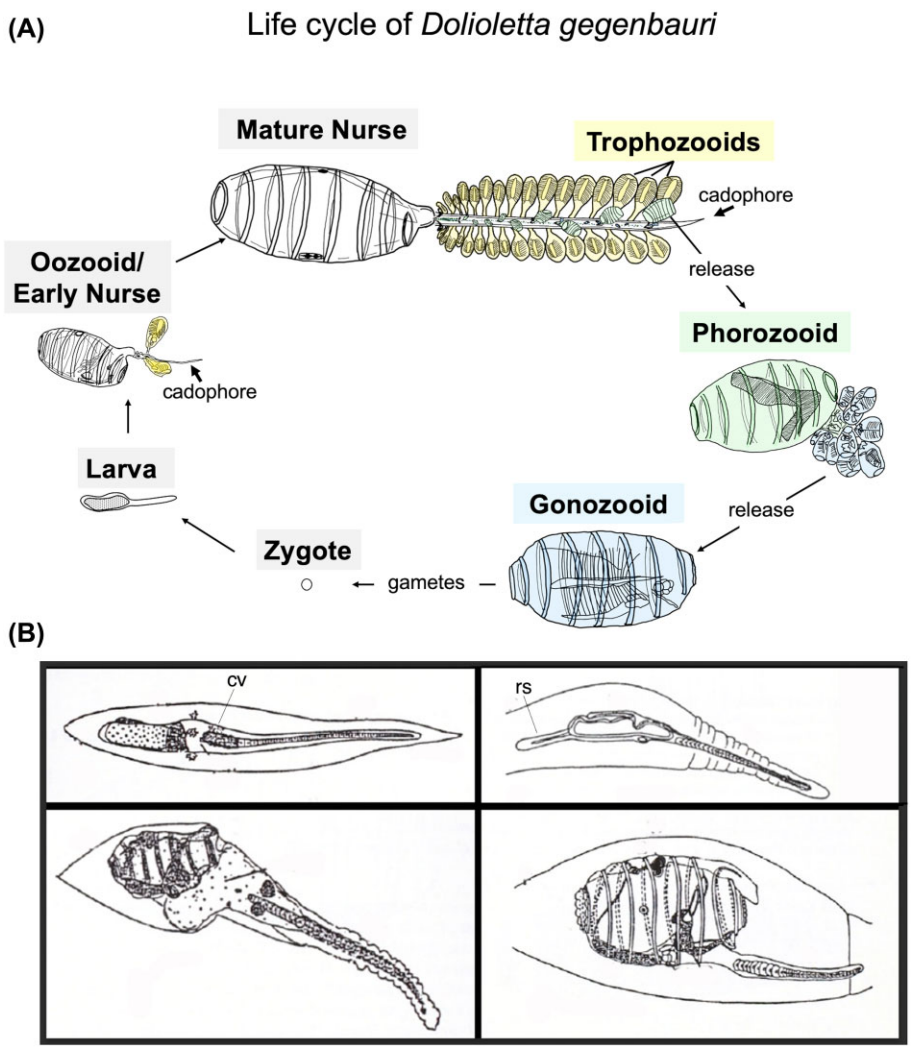

Fig. 4 Doliolid polymorphism and larval diversity. (A) Illustration representing the life history of *Dolioletta gegenbauri* (Quoy and Gaimard 1834) adapted from Walters et al. (2019b). A mature nurse, lacking digestive organs, contains a cadophore with feeding zooids (trophozooids, in yellow) and developing carrier zooids (phorozooids, in green). Mature phorozooids are released, and host developing sexually reproductive gonozooids (in blue). Hermaphroditic gonozooids detach from the phorozooid upon maturity. Once fertilized, a zygote develops into a tadpole-like larva, which undergoes metamorphosis into an oozooid/early nurse. (B) Drawings of diverse doliolid larvae, including early and metamorphosizing *Doliolina mulleri* larvae (left hand panels) along with early and metamorphosizing *Doliolum denticulatum* larvae (right hand panels), adapted from Godeaux 2003 (Godeaux 2003). All representations in A and B lateral views, anterior to the left, cv indicates the caudal vesicle, and rs indicates the rostrum.

of original reports regarding doliolid larvae and fewer describing embryonic development for an extremely limited set of species (Neumann 1906; Neumann et al. 1913; Braconnot 1964; Braconnot 1968; Godeaux 2003). Thus, many developmental stages and fundamental processes remain uncharacterized. One of the best-studied doliolid species is *Dolioletta gegenbauri*. In *D. gegenbauri*, fertilization was suggested to occur externally (Braconnot 1968), while fertilization in *Doliolum nationalis* and *Doliolum denticulatum* was inferred to be internal (Braconnot 1964, 1977). The mode of fertilization has not been proposed for other doliolids. Due to difficulty in culturing doliolid embryos, only a few embryonic stages have been observed, and thus cleavage patterns remain poorly characterized (Braconnot 1971a, 1971b; Godeaux 2003). Unlike the other thaliaceans (salps and pyrosomes), in which embryos develop directly into an adult primary zooid, doliolid embryos develop into tadpole-like larvae that are morphologically similar to ascidian larvae (Fig. 4) (Braconnot 1964; Braconnot 1968). As in ascidian larvae, doliolid larval tails contain a notochord flanked by muscle cells, but they lack a dorsal nerve cord and ventral endodermal strand (Godeaux 1957). Interestingly, development occurs in a follicular envelope that is initially wrapped tightly against the embryo

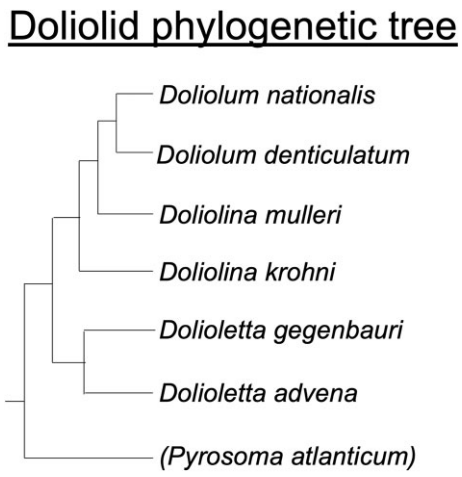

Fig. 5 Doliolid phylogeny. Phylogenetic tree of sequencing data from six doliolid species. Adapted from Garic and Batistic (2022).

but expands to varying degrees as larva develops (Fig. 4B) (Braconnot 1968). In contrast to most solitary ascidians, doliolid larvae initiate metamorphosis prior to tail resorption, leading to the morphogenesis of an adult body (oozooid) in the larval trunk (Braconnot 1964; Braconnot 1968). In this respect, doliolid larvae resemble those of many colonial ascidians (Chia 1977; Karaiskou et al. 2015). Intriguingly, the atrial chamber is formed by bilateral invaginations that fuse to form a single aperture, similar to the process of atrial siphon formation in phlebobranchs. As development proceeds, this adult rudiment expands into the barrel-shaped primary oozooid body, and the tail begins to contract, leading to highly inefficient larval locomotion (Lacalli 1999). Within a short period of time (often less than 2 days), the larval tail is resorbed, completing metamorphosis. The oozooid then hatches from the follicular envelope and begins feeding (Braconnot 1968). Doliolid larval morphology is highly variable (Fig. 4B) (Godeaux 2003). Some species have a caudal vesicle between the trunk and the tail (cv in Fig. 4B, top left). Other species exhibit an anterior protrusion (rostrum) similar to the stolons that serve as a stalk for sessile adult ascidians after settlement and metamorphosis (rs in Fig. 4B, top right).

Doliolid life history

Doliolids have a highly complex, polymorphic life history bearing some similarities to that of other thaliaceans (Figs. 3C and 4A). In addition to a larval stage, doliolids have four distinct adult morphs (oozooids/nurses, trophozooids, phorozooids, and gonozooids). After metamorphosis is complete, the resulting oozooid matures into a nurse that is specialized for locomotion and asexual reproduction. During nurse maturation, an asexually reproductive structure called the cadophore emerges at the dorsal, posterior end of the body. Buds on this stalk-like structure mature into feeding zooids (trophozooids) and asexual, "carrier" zooids (phorozooids). As trophozooids mature, much of the nurse digestive system, including the branchial septum, ventral endostyle, and gut tube degenerate. During early stages of nurse maturation, two rows of buds form on each side of the cadophore and develop into trophozooids, which are characterized by a simplified spoon-shaped body with a large oral aperture and numerous gill slits for feeding. Trophozooids are connected to the colonial vasculature by a short peduncular stalk, presumably allowing distribution of nutrients to the nurse and other developing zooids. Later, two medial rows of buds begin to differentiate into phorozooids, which have the characteristic barrel-shaped doliolid body and are also attached to the cadophore by a peduncular stalk. Cadophores can reach over 15 cm in length and carry hundreds of mature trophozooids and immature phorozooids (Paffenhöfer and Köster 2011). Once the phorozooids are fully mature and beginning to feed, they separate from the colony and nourish developing gonozooid buds on their peduncular stalks (Figs. 4A and 6C). A small colony itself, the free-swimming phorozooid may facilitate the development of dozens of gonozooid buds, as dictated by nutrient availability (Paffenhöfer and Gibson 1999). After the gonozooids have developed a characteristic doliolid barrel-like morphology and are themselves feeding, they are released. Up to eleven juvenile gonozooids can be released from a mature phorozooid in a single day (Paffenhöfer and Gibson 1999). As the hermaphroditic gonozooids feed and mature, they begin to differentiate gonadal tissues. Mature gonozooids have been reported to disperse six eggs per day in laboratory conditions (Walters et al. 2019a).

This summary is based on a few species of doliolids in which a full life history has been characterized. However, isolated stages collected from other, poorly characterized doliolid species exhibit divergent zooid morphologies and may also participate in divergent life history strategies (Godeaux 2003).

The cellular basis of doliolid polymorphism

Doliolid polymorphism can be considered to consist of two morphs produced directly by a fertilized egg (larva and oozooid/nurse) plus three additional morphs that are produced through asexual budding (trophozooids, phorozooids, and gonozooids). The only studies that touch on the cellular origins of these budded zooids

were published over 100 years ago and are based solely on microscopy of fixed specimens (Uljanin 1884; Barrois 1885; Neumann 1906; Sedgwick 1909). As recorded in these early papers, all three of the budded zooids can be traced back to clusters of stem cells (referred to as probuds) that are produced by the nurse. In particular, these probuds appear to arise by "strobilation," pinching off from the end of an elongated organ referred to as a stolon that is positioned on the ventral side of the nurse just posterior to heart. Morphologically similar stolon organs are found in both pyrosome cyathozooids and primary ascidiozooids along with salp oozooids, and all of these organs participate in asexual budding (Fig. 3). However, in *Dolioletta gegenbauri*, the resulting probuds appear to undergo a remarkable migration around the nurse body to the dorsal side and then posteriorly towards the cadophore (Fig. 6A and B). This migration appears to be assisted by cells called phorocytes. Once they arrive at the cadophore, probuds separate into five distinct streams (Fig. 6B and C). These include two lateral streams that mature into trophozooids, two medial streams that mature into phorozooids, and a midline stream of probuds that attach to the developing peduncle of immature phorozooids and subsequently mature into gonozooids (Fig. 6C) (Uljanin 1884; Barrois 1885; Neumann 1906; Sedgwick 1909). The mechanisms dictating probud migration have not been characterized and there is also no data on when or how probuds become differentially specified to form distinct zooids.

Outlook

The doliolids represent a promising model for exploring fundamental questions regarding isogenic polymorphism. The many potential avenues of exploration can be grouped into two major categories of questions and associated approaches.

Questions regarding genetic and cellular mechanisms underlying isogenic polymorphism

Which genes are expressed in a zooid-specific manner? How is zooid-specific gene expression regulated? How were developmental gene networks altered to permit redeployment of one genome for multiple developmental programs? Do some zooids contain novel cell types, tissues, or organs, or are zooid-specific structures produced by remodeling of ancestral structures? How were ancestral cell lineages redeployed to generate novel morphs? At what point does the fate of stem cells used for asexual budding of distinct morphs diverge? What signals or cues drive this divergence?

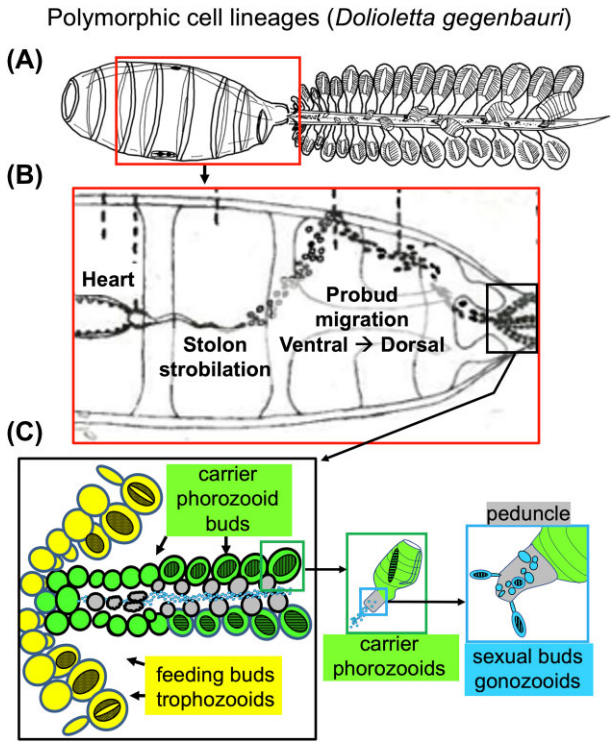

Fig. 6 Distinct probud lineages produce feeding, carrier, and sexual zooids. (**A**) Depiction of a mature *D. gegenbauri* nurse, red box indicates the posterior trunk region as detailed in the next panel. (**B**) Probuds produced by a ventral stolon migrate to the dorsal, posterior cadophore, adapted from Barrois (1885). Black box indicates the proximal end of the cadophore, as detailed in the next panel. (**C**) In the proximal end of the cadophore, there are three spatially distinct lines of maturing probuds, including two lateral lines of maturing trophozooid buds (in yellow), two medial lines of maturing phorozooid buds (in green), and a midline of gonozooid probuds (in blue). Boxed regions indicate the peduncle of a maturing phorozooid, as detailed in the last two panels. Note that gonozooid buds (blue) produced by the nurse stolon attach to this peduncle and then begin to mature once the phorozooid is released (last panel).

Approaches to address these questions

Studies of isogenic polymorphism in the siphonophores (Dunn and Wagner 2006; Siebert et al. 2015; Munro et al. 2018; Mah and Dunn 2024) provide a roadmap for studying related processes in the doliolids. In particular, phylogenetically informed analysis of RNA sequencing data can be used to identify shared zooid-specific genes in multiple, related polymorphic species. These results, particularly zooid-specific transcription or signaling factor gene expression, can guide efforts to identify potential regulatory pathways mediating zooid-specific expression and to reconstruct underlying gene regulatory networks. These efforts could be greatly enhanced by use of single-cell resolution RNA sequencing of isolated cells or tissues, referred to as scRNA-seq or spatial transcriptomics, respectively (Klein et al. 2015;

Cao et al. 2019; Rao et al. 2021; Jiang et al. 2024). These efforts could be further enhanced by deployment of single-cell resolution chromatin profiling assays such as scATAC-seq (Sinha et al. 2021; Grandi et al. 2022). The presence of spatially and temporally distinct probud lineages in the nurse cadophore represents an extraordinary opportunity to deploy these methods. In particular, single-cell RNA sequencing of probuds isolated from different regions along the migratory route from the stolon to the cadophore (Fig. 6B) or in medial to lateral regions of the cadophore (Fig. 6C) would permit the reconstruction of gene expression trajectories driving divergent specification of trophozooid vs. phorozooid vs. gonozooid buds. High-resolution spatial transcriptomics could achieve a similar result.

Ideally, in-lab culturing would be employed to generate samples required for sequencing analyses and comparative embryology. Currently, *Dolioletta gegenbauri* is the only doliolid species for which robust in-lab culturing methods have been developed (Walters et al. 2019b). Modiflying and refining these methods to culture additional doliolid species will be essential. In the meantime, multiple species of nurses with sufficiently intact cadophores can be readily collected from the plankton and subjected to RNA-seq or scRNA-seq analyses. Genomic sequencing to refine doliolid taxonomic relationships and development of appropriate tools to analyze this RNA-sequencing data in relation to these phylogenetic data will be essential to this effort (Mah and Dunn 2024). We have initiated this project through genomic sequencing and zooid-specific RNA-sequencing in the species *Dolioletta gegenbauri*.

Questions regarding the evolutionary acquisition and divergence of isogenic polymorphism

What form of polymorphism was present in the most recent common ancestor of the thaliaceans? How was polymorphism acquired in a presumably colonial ascidian to produce this common ancestor? How did a more elaborate mode of polymorphism arise in doliolids (or was this trait lost in the other thaliaceans)? How did polymorphism diverge within the doliolids? How did selection drive the acquisition and diversification of polymorphism? How has antagonistic selection been resolved in doliolids and other thaliaceans?

Approaches to address these questions

Comparative RNA-sequencing, as detailed in the previous section would provide a powerful platform for deciphering many of these evolutionary questions. In order to fully address questions regarding initial emergence of polymorphism in either thaliaceans or doliolids, this effort would have to be extended to include multiple salp and pyrosome species along with at least one colonial phlebobranch species that can serve to root these analyses in regards to the presumptive, pre-polymorphic ancestor of the thaliaceans. Comparative microanatomical observations of budding in colonial aplousobranch ascidians, salps, pyrosomes, and doliolids indicate that they share some potentially homologous features. In particular, as summarized by Alié et al. (2021), they all appear to produce asexually reproductive strands by outpocketing of the pharynx. Thus, Alié et al. refer to this mode as "pharyngeal budding" and point out that although these structures are called "stolons" in the classic literature, they are distinct from the stolonic budding observed in other ascidians. Instead, as proposed numerous times in the classic literature, thaliacean budding may be derived from an ancestral form of epicardial budding that is retained in many colonial aplousobranch ascidians (Bonnevie 1896; Brien 1928; Berrill 1935; Godeaux 1957; Ivanova-Kazas 1967). The placement of both aplousobranch and thaliaceans within a large, polyphyletic clade of phlebobranchs may reflect the presence of epicardial budding in a colonial phlebobranch ancestor that was subsequently lost in solitary phlebobranch lineages (Fig. 2). Exploring this model will require in-depth comparative studies of epicardial budding, including high-resolution delineation of gene expression, cell types, and molecular pathways in a range of thaliaceans and colonial phlebobranchs. In considering the emergence of a more elaborate polymorphic life history in the doliolids, it is striking that all thaliaceans have both sexual and asexual adult morphs. Thus, it may be that the shared thaliacean ancestor had a relatively simple, three-part life history, including a tadpole larval stage (as seen in doliolids and most tunicates), a primary zooid that reproduces asexually through epicardial budding (as seen in all thaliaceans), and secondary sexual zooids (as seen in salps). According to this scenario, the salps and pyrosomes lost the tadpole larval stage and the pyrosomes also acquired two additional zooids, a secondary asexual zooid and a tertiary composite (sexual/asexual) zooid. In relation to doliolids, this scenario requires the acquisition of three key divergent traits: (1) a novel feeding zooid (the trophozooids), (2) an additional novel zooid specialized for carrying and dispersing sexual buds (the phorozooids), and (3) a composite mode of stolonic budding in which the primary zooid (the nurse) serves as the origin for carrier, feeding,and sexual buds. The comparative genomic and RNA sequencing efforts outlined above would provide the basis for distinguishing between

this scenario and many potential alternative models regarding the divergence of polymorphism within the thaliaceans.

Deciphering the potential role of antagonistic selection on morph-specific trait acquisition would involve comparative microanatomical studies of zooid morphology across multiple doliolid species (as mentioned in the previous section) along with a more in-depth analysis examining trait variation among individuals. Complementary sequencing studies could be used to assess genetic correlations between traits. These data could be used to begin testing predictions regarding the possible influence of antagonistic selection on genetic correlation, particularly in regards to morph-specific traits (Goedert and Calsbeek 2019). The development of higher throughput in-lab culturing would be required for directly assessing potential antagonistic selection associated with morph-specific traits across a suitably high number of individuals (Goedert and Calsbeek 2019).

Doliolids and their thaliacean cousins are an enigma, a fascinating living puzzle that we only now have the tools to decipher. Exploring the elaborate life history of these fragile and beautiful animals has the potential to provide fundamental insights into the architecture of the gene networks that program development and how these networks are restructured to drive diversification within our own chordate phyla. Unraveling this puzzle also promises to illuminate fundamental principles of antagonistic selection that impact a much broader group of organisms with sexual or life history dimorphism. Additionally, a deeper knowledge of thaliacean life history will inform our understanding of the critical role these organisms play in marine ecology. It is not clear how many of the highly diverse doliolid and other thaliacean species recorded in the classic literature remain extant. Some of these species may already have been lost due to global warming and rapid degradation of marine environments. Thus, there is an urgent need to study and conserve these obscure and unique organisms before they are lost.

Author contributions

C.J. Pickett: Writing – Original draft. Joseph Ryan: Writing – review & editing. Bradley Davidson: Conceptualization, Funding Acquisition, Writing – review & editing

Conflict of interest

The authors have no conflict of interests associated with this paper.

Data availability

There are no new data associated with this article.

References

Abouheif E. 2021. Ant caste evo-devo: it's not all about size. Trends Ecol Evol 36:668–70. https://doi.org/10.1016/j.tree.2021.04.002

Aguirre JD, Blows MW, Marshall DJ. 2014. The genetic covariance between life cycle stages separated by metamorphosis. Proc Biol Sci 281:20141091. https://doi.org/ARTN 20141091 10.1098/rspb.2014.1091

Alié A, Hiebert LS, Scelzo M, Tiozzo S. 2021. The eventful history of nonembryonic development in tunicates. J Exp Zool Pt B 336:250–66. https://doi.org/10.1002/jez.b.22940

Bardi J, Marques AC. 2007. Taxonomic redescription of the Portuguese man-of-war, (Cnidaria, Hydrozoa, Siphonophorae, Cystonectae) from Brazil. Iheringia, Sér. Zool. 97:425–33. https://doi.org/10.1590/S0073-47212007000400011

Barrois J. 1885. Recherches sur le cycle génétique et le bourgeonnement de l'Anchinie. J Anat Physiol 21:193–267.

Beklemishev VN, MacLennan JM, Kabata Z. 1969. Principles of comparative anatomy of invertebrates. Edinburg: Oliver & Boyd.

Berrill NJ. 1935. IX—Studies in tunicate development Part IV—Asexual reproduction. Philos Trans R Soc Lond B Biol Sci 225:327–79.

Bonasio R. 2014. The role of chromatin and epigenetics in the polyphenisms of ant castes. Brief Funct Genom 13:235–45. https://doi.org/10.1093/bfgp/elt056

Bone Q. 1998. The biology of pelagic tunicates. Oxford: Oxford University Press.

Bonnevie K. 1896. Om Knopskydningen hos" Distaplia magnilarva" og" Pyrosoma elegans," af Kristine Bonnevie…-On gemmation in" Distaplia magnilarva," and" Pyrosoma elegans,". In: Bonnevie K, editor. Norway: Grøndahl.

Braconnot J. 1964. Sur Le Developpement De La Larve De Doliolum Denticulatum Q Et G. C R Hebd Seances Acad Sci 259:4361–2.

Braconnot J. 1977. SUR LE CYCLE SEXUE CHEZ LE TUNICIER PELAGIQUE DOLIOLUM NATIONALIS, BORG, 1893, AVEC LA PREMIERE DESCRIPTION DU STADE OOZOIDE(https://archimer.ifremer.fr/doc/00000/3871/).

Braconnot JC. 1968. Larval Development of Pelagic Tunicier Doliolide Doliolum (Dolioletta) Gegenbauri Ulj 1884. Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D 267:629.

Braconnot J-C. 1971a. Contribution a l'etude biologique et ecolo-gique des tuniciers pelagiques salpides et doliolides. 1. Hydrologic et ecologic des Salpides. Vie Et Milieu 22:257–86.

Braconnot J-C. 1971b. CONTRIBUTION A L'ÉTUDE BIOLOGIQUE ET ÉCOLOGIQUE DES TUNICIERS PÉLAGIQUES SALPIDES ET DOLIOLIDES II. _ ÉCOLOGIE DES DOLIOLIDES, BIOLOGIE DES DEUX GROUPES. Vie Et Milieu 437–67.

Braun K, Leubner F, Stach T. 2020. Phylogenetic analysis of phenotypic characters of Tunicata supports basal Appendicularia and monophyletic Ascidiacea. Cladistics 36:259–300. https://doi.org/10.1111/cla.12405

Brien P. 1928. Contribution a l'étude de l'embryogénèse et de la blastogénèse des salpes. Belgium: L'Université des Bruxelles.
Bump P, Lubeck L. 2023. Marine invertebrates one cell at a time: insights from single-cell analysis. Integr Comp Biol 63:999–1009.
Cao C, Lemaire LA, Wang W, Yoon PH, Choi YA, Parsons LR, Matese JC, Wang W, Levine M, Chen K. 2019. Comprehensive single-cell transcriptome lineages of a proto-vertebrate. Nature 571:349–54. https://doi.org/10.1038/s41586-019-1385-y
Carre C. 1967. Le Developpement Larvaire Dabylopsis Tetragona Otto 1823 (Siphonophore Calycophore Abylidae). Cahiers De Biologie Marine 8:185.
Carre C. 1969. On Genus Lilyopsis Chun 1885 with a Redescription of Species Lilyopsis Rosea Chun 1885 (Siphonophora Prayinae) and a Description of Its Calyconula Phase. Cahiers De Biologie Marine 10:71.
Carre C, Carre D. 1991. A complete life-cycle of the calycophoran siphonophore *Muggiaea kochi* (Will) in the laboratory, under different temperature conditions—ecological implications. Phil Trans R Soc Lond B 334:27–32. https://doi.org/10.1098/rstb.1991.0095
Carre D. 1967. Larval Development in 2 Siphonophores Lensia Conoidea (Calycophore) and Forskalia Edwardsi (Physonecte). Cahiers De Biologie Marine 8:233.
Chia FS, Rice ME. 1977. Settlement and metamorphosis of marine invertebrate larvae. Proceedings of the American Zoological Society Meeting, Toronto, Canada (New York, Elsevier, 1978).
Church SH, Siebert S, Bhattacharyya P, Dunn CW. 2015. The histology of (Hydrozoa: Siphonophora). J Exp Zool Pt B 324:435–49. https://doi.org/10.1002/jez.b.22629
Cutter AD. 2023. Speciation and development. Evol Dev 25:289–327. https://doi.org/10.1111/ede.12454
Davidson EH. 2010. The regulatory genome: gene regulatory networks in development and evolution. Netherlands: Elsevier.
Debiasse MB, Colgan WN, Harris L, Davidson B, Ryan JF. 2020. Inferring tunicate relationships and the evolution of the tunicate Hox cluster with the genome of *Corella inflata*. Genome Biol Evolut 12:948–64. https://doi.org/10.1093/gbe/evaa060
Deibel D. 1985. Blooms of the pelagic tunicate, *Dolioletta gegenbauri*: are they associated with Gulf-Stream frontal eddies. J Mar Res 43:211–36. https://doi.org/10.1357/002224085788437307
Deibel D, Lowen B. 2012. A review of the life cycles and life-history adaptations of pelagic tunicates to environmental conditions. ICES J Mar Sci 69:358–69. https://doi.org/10.1093/icesjms/fsr159
Deibel D, Paffenhofer G-A. 2009. Predictability of patches of neritic salps and doliolids (Tunicata, Thaliacea). J Plankton Res 31:1571–9. https://doi.org/10.1093/plankt/fbp091
Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439:965–8. https://doi.org/10.1038/nature04336
Delsuc F, Philippe H, Tsagkogeorga G, Simion P, Tilak MK, Turon X, López-Legentil S, Piette J, Lemaire P, Douzery EJP. 2018. A phylogenomic framework and timescale for comparative studies of tunicates. BMC Biol 16:39. https://doi.org/ARTN 39 10.1186/s12915-018-0499-2
Dunn CW, Wagner GP. 2006. The evolution of colony-level development in the Siphonophora (Cnidaria:Hydrozoa). Dev Genes Evol 216:743–54. https://doi.org/10.1007/s00427-006-0101-8
Ferrández-Roldán A, Fabregà-Torrus M, Sánchez-Serna G, Duran-Bello E, Joaqui´n-Llui´s M, Bujosa P, Plana-Carmona M, Garcia-Fernàndez J, Albalat R, Cañestro C. 2021. Cardiopharyngeal deconstruction and ancestral tunicate sessility. Nature 599:431–5. https://doi.org/10.1038/s41586-021-04041-w
Fiuza U-M, Lemaire P. 2021. Mechanical and genetic control of ascidian endoderm invagination during gastrulation. Semin Cell Dev Biol 120:108–18.
Fodor ACA, Powers MM, Andrykovich K, Liu J, Lowe EK, Brown CT, Di Gregorio A, Stolfi A, Swalla BJ. 2021. The degenerate tale of ascidian tails. Integr Comp Biol 61:358–69. https://doi.org/10.1093/icb/icab022
Frischer ME, Lamboley LM, Walters TL, Brandes JA, Arneson E, Lacy LE, López-Figueroa NB, Rodríguez-Santiago ÁE, Gibson DM. 2021. Selective feeding and linkages to the microbial food web by the doliolid. Limnol Oceanogr 66:1993–2010. https://doi.org/10.1002/lno.11740
Garic R, Batistic M. 2022. *Dolioletta advena* sp. nov., a new species of doliolid (Tunicata, Thaliacea) from the Adriatic Sea. Diversity (Basel) 14:959. https://doi.org/ARTN 959 10.3390/d14110959
Gibson DM. 2002. Asexual reproduction of the doliolid, *Dolioletta gegenbauri* Uljanin (Tunicata, Thaliacea). J Plankton Res 24:703–12. https://doi.org/10.1093/plankt/24.7.703
Gibson DM, Paffenhöfer GA. 2000. Feeding and growth rates of the doliolid, *Dolioletta gegenbauri* Uljanin (Tunicata, Thaliacea). J Plankton Res 22:1485–1500. https://doi.org/10.1093/plankt/22.8.1485
Gilbert E, Teeling C, Lebedeva T, Pedersen S, Chrismas N, Genikhovich G, Modepalli V. 2022. Molecular and cellular architecture of the larval sensory organ in the cnidarian *Nematostella vectensis*. Development 149:dev200833.
Godeaux J. 1957. Contribution a la connaissance des thaliaces (Pyrosome et Doliolum): these. Belgium: Universitte de Liege, Faculte des Sciences. https://books.google.com/books?id=9L9bxgEACAAJ
Godeaux JE. 2003. History and revised classification of the order Cyclomyaria (Tunicata, Thaliacea, Doliolida). Bulletin De L'institut Royal Des Sciences Naturelles De Belgique, Biologie 73:191–222.
Godeaux JEA. 1998. The genus *Doliolina* (Thaliacea: Doliolida). J Plankton Res 20:1757–66. https://doi.org/10.1093/plankt/20.9.1757
Godeaux JEA, Harbison GR. 2003. On some pelagic Doliolid tunicates (Traliacea, Doliolida) collected by a submersible off the eastern NorthAmerican coast. Bull Mar Sci 72:589–612. <Go to ISI>://WOS:000187026500001
Goedert D, Calsbeek R. 2019. Experimental evidence that metamorphosis alleviates genomic conflict. Am Nat 194:356–66. https://doi.org/10.1086/704183
Grandi FC, Modi H, Kampman L, Corces MR. 2022. Chromatin accessibility profiling by ATAC-seq. Nat Protoc 17:1518–52. https://doi.org/10.1038/s41596-022-00692-9
Harvell CD. 1991. Coloniality and inducible polymorphism. Am Nat 138:1–14. https://doi.org/10.1086/285201

Harvell CD. 1994. The evolution of polymorphism in colonial invertebrates and socialiInsects. Q Rev Biol 69:155–85. https://doi.org/10.1086/418538

Herpin A, Schartl M. 2015. Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpators. EMBO Rep 16:1260–74. https://doi.org/10.15252/embr.201540667

Hiebert LS, Vieira LM, Tiozzo S, Simpson C, Grosberg RK, Migotto AE, Morandini AC, Brown FD. 2021. From the individual to the colony: marine invertebrates as models to understand levels of biological organization. J Exp Zool Pt B 336:191–7. https://doi.org/10.1002/jez.b.23044

Ivanova-Kazas OM. 1967. Reproduction asexuée et cycle évolutif de l'ascidie *Distaplia unigermis*. Cahiers De Biologie Marine 8:21–62.

Jiang A, Han K, Wei J, Su X, Wang R, Zhang W, Liu X, Qiao J, Liu P, Liu Q et al. 2024. Spatially resolved single-cell atlas of ascidian endostyle provides insight into the origin of vertebrate pharyngeal organs. Sci Adv 10:eadi9035. https://doi.org/10.1126/sciadv.adi9035

Karaiskou A, Swalla BJ, Sasakura Y, Chambon J-P. 2015. Metamorphosis in solitary ascidians. Genesis 53:34–47. https://doi.org/10.1002/dvg.22824

Klein AM, Mazutis L, Akartuna I, Tallapragada N, Veres A, Li V, Peshkin L, Weitz DA, Kirschner MW. 2015. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell 161:1187–201. https://doi.org/10.1016/j.cell.2015.04.044

Lacalli TC. 1999. Tunicate tails, stolons, and the origin of the vertebrate trunk. Biol Rev 74:177–98. https://doi.org/10.1017/S0006323199005319

Lambert G. 2005. Ecology and natural history of the protochordates. Can J Zool 83:34–50. https://doi.org/10.1139/Z04-156

Liang C, Forrest ARR, Wagner GP. 2015. The statistical geometry of transcriptome divergence in cell-type evolution and cancer. Nat Commun 6:6066.

Lidgard S, Carter MC, Dick MH, Gordon DP, Ostrovsky AN. 2012. Division of labor and recurrent evolution of polymorphisms in a group of colonial animals. Evol Ecol 26:233–57. https://doi.org/10.1007/s10682-011-9513-7

Lilly LE, Suthers IM, Everett JD, Richardson AJ. 2023. A global review of pyrosomes: shedding light on the ocean's elusive gelatinous "fire-bodies". Limnol Oceanogr Lett 8:812–29. https://doi.org/10.1002/lol2.10350

Linksvayer TA, Fewell JH, Gadau J, Laubichler MD. 2012. Developmental evolution in social insects: regulatory networks from genes to societies. J Exp Zool Pt B 318:159–69. https://doi.org/10.1002/jez.b.22001

Mackie GO. 1960. The structure of the nervous system in velella. Q J Microsc Sci 101:119.

Macrander J, Brugler MR, Daly M. 2015. A RNA-seq approach to identify putative toxins from acrorhagi in aggressive and non-aggressive polyps. BMC Genomics 16:221. https://doi.org/ARTN 221 10.1186/s12864-015-1417-4

Mah JL, Dunn CW. 2024. Cell type evolution reconstruction across species through cell phylogenies of single-cell RNA sequencing data. Nat Ecol Evol 8:325–38. https://doi.org/10.1038/s41559-023-02281-9

Martí-Solans J, Godoy-Marín H, Diaz-Gracia M, Onuma TA, Nishida H, Albalat R, Cañestro C. 2021. Massive gene loss and function shuffling in appendicularians stretch the boundaries of chordate Wnt family evolution. Front Cell Dev Biol 9:700827. https://doi.org/10.3389/fcell.2021.700827

Munro C, Siebert S, Zapata F, Howison M, Damian-Serrano A, Church SH, Goetz FE, Pugh PR, Haddock SHD, Dunn CW. 2018. Improved phylogenetic resolution within Siphonophora (Cnidaria) with implications for trait evolution. Mol Phylogenet Evol 127:823–33. https://doi.org/10.1016/j.ympev.2018.06.030

Munro C, Vue Z, Behringer RR, Dunn CW. 2019. Morphology and development of the Portuguese man of war, *Physalia physalis*. Sci Rep 9:15522. https://doi.org/10.1038/s41598-019-51842-1

Munro C, Zapata F, Howison M, Siebert S, Dunn CW. 2022. Evolution of gene expression across species and specialized zooids in Siphonophora. Mol Biol Evol 39:msac027. https://doi.org/ARTN msac027 10.1093/molbev/msac027

Musser JM, Schippers KJ, Nickel M, Mizzon G, Kohn AB, Pape C, Ronchi P, Papadopoulos N, Tarashansky AJ, Hammel JU et al. 2021. Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. Science 374:717–23.

Neumann G. 1906. Doliolum. Wissenschaftliche Ergebnisse Der Deutschen Tiefsee Expedition 1898–1899. (12 (II)), 97–243, 214 pl., 241 map(https://www.biodiversitylibrary.org/bibliography/2171).

Neumann G, Thiele J, Mortensen T. 1913. Die pyrosomen und dolioliden der deutschen südpolar-expedition 1901-1903. Germany: Verlag von Georg Reimer.

Onuma TA, Nishida H. 2022. Developmental biology of the larvacean *Oikopleura dioica*: Genome resources, functional screening, and imaging. Dev Growth Differ 64:67–82. https://doi.org/10.1111/dgd.12769

Paffenhöfer G-A, Gibson D. 1999. Determination of generation time and asexual fecundity of doliolids (Tunicata, Thaliacea). J Plankton Res 21:1183–9. https://doi.org/10.1093/plankt/21.6.1183

Paffenhöfer G-A. 2013. A hypothesis on the fate of blooms of doliolids (Tunicata, Thaliacea). J Plankton Res 35:919–24. https://doi.org/10.1093/plankt/fbt048

Paffenhöfer G-A, Koster M. 2011. From one to many: on the life cycle of *Dolioletta gegenbauri* Uljanin (Tunicata, Thaliacea). J Plankton Res 33:1139–45. https://doi.org/10.1093/plankt/fbr001

Paris M, Laudet V. 2008. The history of a developmental stage: metamorphosis in chordates. Genesis 46:657–72. https://doi.org/10.1002/dvg.20443

Peter IS, Davidson EH. 2011. Evolution of gene regulatory networks controlling body plan development. Cell 144:970–85. https://doi.org/10.1016/j.cell.2011.02.017

Piette J, Lemaire P. 2015. Thaliaceans, the neglected pelagic relatives of ascidians: a developmental and evolutionary enigma. Q Rev Biol 90:117–45. https://doi.org/10.1086/681440

Plachetzki DC, Sabrina Pankey M, Johnson BR, Ronne EJ, Kopp A, Grosberg RK. 2014. Gene co-expression modules underlying polymorphic and monomorphic zooids in the colonial hydrozoan. Integr Comp Biol 54:276–83. https://doi.org/10.1093/icb/icu080

Pyenson BC, Rehan SM. 2024. Gene regulation supporting sociality shared across lineages and variation in complexity. Genome 67:99–108. https://doi.org/10.1139/gen-2023-0054

Quoy J, Gaimard J. 1834. Voyage de De ´couvertes de l'Astrolabe. Pendant les annees 1826-1829. France: Tastu, Paris. p. 1–954.
Rao A, Barkley D, França GS, Yanai I. 2021. Exploring tissue architecture using spatial transcriptomics. Nature 596:211–20. https://doi.org/10.1038/s41586-021-03634-9
Richardson MK. 2022. Theories, laws, and models in evo-devo. J Exp Zool Pt B 338:36–61. https://doi.org/10.1002/jez.b.23096
Robison BH, Raskoff KA, Sherlock RE. 2005. Ecological substrate in midwater: *D oliolula equus*, a new mesopelagic tunicate. J. Mar. Biol. Ass. 85:655–63. https://doi.org/10.1017/S0025315405011586
Sanders SM, Cartwright P. 2015. Interspecific differential expression analysis of RNA-Seq data yields insight into life cycle variation in hydractiniid hydrozoans. Genome Biol Evol 7:2417–31. https://doi.org/10.1093/gbe/evv153
Sanders SM, Shcheglovitova M, Cartwright P. 2014. Differential gene expression between functionally specialized polyps of the colonial hydrozoan *Hydractinia symbiolongicarpus* (Phylum Cnidaria). BMC Genomics 15:406. https://doi.org/Artn 406 10.1186/1471-2164-15-406
Schack CR, Gordon DP, Ryan KG. 2019. Modularity is the mother of invention: a review of polymorphism in bryozoans. Biol Rev 94:773–809. https://doi.org/10.1111/brv.12478
Sedgwick A. 1909. A student's textbook of zoology. London: Swan Sonnenschein and Company.
Shubin N, Tabin C, Carroll S. 2009. Deep homology and the origins of evolutionary novelty. Nature 457:818–23. https://doi.org/10.1038/nature07891
Siebert S, Goetz FE, Church SH, Bhattacharyya P, Zapata F, Haddock SHD, Dunn CW. 2015. Stem cells in *Nanomia bijuga* (Siphonophora), a colonial animal with localized growth zones. Evodevo 6:22. https://doi.org/10.1186/s13227-015-0018-2
Siebert S, Robinson MD, Tintori SC, Goetz F, Helm RR, Smith SA, Shaner N, Haddock SHD, Dunn CW. 2011. Differential gene expression in the siphonophore *Nanomia bijuga* (Cnidaria) assessed with multiple next-generation sequencing workflows. PLoS One 6:e22953. https://doi.org/ARTN e22953 10.1371/journal.pone.0022953
Simpson C, Jackson JBC, Herrera-Cubilla A. 2017. Evolutionary determinants of morphological polymorphism in colonial animals. Am Nat 190:17–28. https://doi.org/10.1086/691789
Sinha S, Satpathy AT, Zhou W, Ji H, Stratton JA, Jaffer A, Bahlis N, Morrissy S, Biernaskie JA. 2021. Profiling chromatin accessibility at single-cell resolution. Genomics Proteomics Bioinformatics 19:172–90.
Stach T, Turbeville JM. 2002. Phylogeny of Tunicata inferred from molecular and morphological characters. Mol Phylogenet Evol 25:408–28. https://doi.org/Pii S1055-7903(02)00305-6
Stach T, Winter J, Bouquet J-M, Chourrout D, Schnabel R. 2008. Embryology of a planktonic tunicate reveals traces of sessility. Proc Natl Acad Sci USA 105:7229–34. https://doi.org/10.1073/pnas.0710196105
Stern DL. 2000. Evolutionary developmental biology and the problem of variation. Evolution 54:1079–91. https://doi.org/10.1111/j.0014-3820.2000.tb00544.x
Swalla BJ, Cameron CB, Corley LS, Garey JR. 2000. Urochordates are monophyletic within the deuterostomes. Syst Biol 49:52–64.
Takahashi K, Ichikawa T, Fukugama C, Yamane M, Kakehi S, Okazaki Y, Kubota H, Furuya K. 2015. In situ observations of a doliolid bloom in a warm water filament using a video plankton recorder: Bloom development, fate, and effect on biogeochemical cycles and planktonic food webs. Limnol Oceanogr 60:1763–80. https://doi.org/10.1002/lno.10133
Tanay A, Sebé-Pedrós A. 2021. Evolutionary cell type mapping with single-cell genomics. Trends Genet 37:919–32.
Truman JW. 2019. The evolution of insect metamorphosis. Curr Biol 29:R1252–68. https://doi.org/10.1016/j.cub.2019.10.009
Tsagkogeorga G, Turon X, Hopcroft RR, Tilak MK, Feldstein T, Shenkar N, Loya Y, Huchon D, Douzery EJP, Delsuc F. 2009. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evol Biol 9:187. https://doi.org/Artn 187 10.1186/1471-2148-9-187
Uljanin VN. 1884. Die arten der gattung Doliolum im golfe von Neapel und den angrenzenden meeresabschnitten(Vol. 10). Germany: W. Engelmann.
Walters TL, Gibson DM, Frischer ME. 2019a. Cultivation of the marine pelagic tunicate (Uljanin 1884) for experimental studies. J Vis Exp 150. https://doi.org/ARTN e59832 10.3791/59832
Walters TL, Lamboley LM, López-Figueroa NB, Rodríguez-Santiago ÁE, Gibson DM, Frischer ME. 2019b. Diet and trophic interactions of a circumglobally significant gelatinous marine zooplankter, *Dolioletta gegenbauri* (Uljanin, 1884). Mol Ecol 28:176–89. https://doi.org/10.1111/mec.14926
Williams TM, Carroll SB. 2009. Genetic and molecular insights into the development and evolution of sexual dimorphism. Nat Rev Genet 10:797–804. https://doi.org/10.1038/nrg2687
Yamakawa S, Morino Y, Kohtsuka H, Wada H. 2019. Retinoic acid signaling regulates the metamorphosis of feather stars (Crinoidea, Echinodermata): insight into the evolution of the animal life cycle. Biomolecules 10:37. https://doi.org/10.3390/biom10010037
Yokobori S-I, Oshima T, Wada H. 2005. Complete nucleotide sequence of the mitochondrial genome of *Doliolum nationalis* with implications for evolution of urochordates. Mol Phylogenet Evol 34:273–83. https://doi.org/10.1016/j.ympev.2004.10.002
Zhang TJ, Xu YC, Imai KR, Fei T, Wang GL, Dong B, Yu TW, Satou Y, Shi WY, Bao ZR. 2020. A single-cell analysis of the molecular lineage of chordate embryogenesis. Sci Adv 6:eabc4773. https://doi.org/ARTN eabc4773 10.1126/sciadv.abc4773