REVIEW / SYNTHÈSE

Historical introduction, overview, and reproductive biology of the protochordates[1]

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Abstract: This issue of the *Canadian Journal of Zoology* exhaustively reviews most major aspects of protochordate biology by specialists in their fields. Protochordates are members of two deuterostome phyla that are exclusively marine. The Hemichordata, with solitary enteropneusts and colonial pterobranchs, share a ciliated larva with echinoderms and appear to be closely related, but they also have many chordate-like features. The invertebrate chordates are composed of the exclusively solitary cephalochordates and the tunicates with both solitary and colonial forms. The cephalochordates are all free-swimming, but the tunicates include both sessile and free-swimming forms. Here I explore the history of research on protochordates, show how views on their relationships have changed with time, and review some of their reproductive and structural traits not included in other contributions to this special issue.

Résumé : Le numéro courant de la *Revue canadienne de zoologie* présente une synthèse exhaustive par des spécialistes des principaux aspects de la biologie des protochordés. Les protochordés appartiennent à deux phylums exclusivement marins de deutérostomiens. Les hémichordés qui contiennent les entéropneustes solitaires et les ptérobranches coloniaux ont, comme les échinodermes, des larves ciliées et semblent leur être apparentés, tout en ayant de nombreuses caractéristiques semblables à celles des chordés. Les chordés invertébrés comprennent les céphalochordés qui sont toujours solitaires et les tuniciers qui possèdent des formes solitaires et coloniales. Les céphalochordés nagent librement dans l'eau, alors que les tuniciers sont représentés par des formes nageuses et sessiles. Ma contribution retrace l'histoire de la recherche sur les protochordés, elle montre comment les relations entre les groupes ont été interprétées différemment au cours des années et elle fait le point sur certaines caractéristiques reproductives et structurales qui ne sont pas traitées dans les autres présentations de cette série.

[Traduit par la Rédaction]

Historical overview

This issue of the *Canadian Journal of Zoology* is devoted to protochordates, an intriguing and somewhat disparate group of marine invertebrates. These reviews convey the excitement of the current renaissance of interest in these members of two deuterostome phyla: Hemichordata and the invertebrate members of the Chordata. Molecular data places the hemichordates closer to the echinoderms than the chordates, but we treat the groups here as they have been considered historically. In this issue, Ruppert (2005) critically considers the relationship between these organisms in morphological

[1]This review is one of a series dealing with aspects of the biology of the Protochordata. This series is one of several virtual symposia on the biology of neglected groups that will be published in the Journal from time to time.

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terms, while Zeng and Swalla (2005) examines their molecular phylogeny and suggests that tunicates be placed in their own phylum. The designation hemichordate was by Bateson (1885), who considered their development sufficiently chordate-like to place them in the same phylum as the other chordates, but Hyman (1959) and most modern authors regard them as a separate phylum. There are fewer than 100 species of hemichordates, which include the solitary class Enteropneusta with about 70 species of benthic organisms living in soft substrates and the class Pterobranchia with only 21 species of tubiculous and colonial organisms living on shells and other hard substrates (Ruppert and Barnes 1994).

The invertebrate chordates include two subphyla: Tunicata and Cephalochordata. Since the cephalochordates lack the most anterior portions of the vertebrate nervous system, the term Acrania has also been applied to them. The cephalochordates are a small group with only about 30 species of free-living benthic organisms that live in coarse sands throughout the world (reviewed by Lambert 2005). They were considered mollusks until their fish-like nature was discovered in the 19th century. The Tunicata (often incorrectly called the Urochordata; P. Kott, personal communication) are the most prevalent protochordates and include both free-swimming and fixed classes. With few exceptions, they are

all hermaphrodites. Thaliaceans and appendicularians (sometimes called larvaceans) are free-swimming, whereas ascidians and sorberaceans (Monniot and Monniot 1990; but see Kott 1989b, 1998) are generally anchored as adults. Thaliaceans include pyrosomid colonies as well as solitary and colonial doliolids and salps. Life cycles reach extremes of complexity in this group. Like the thaliaceans, appendicularians are also planktonic but lack colonial forms and have simple life cycles (Ruppert and Barnes 1994). Although pelagic tunicates include relatively few species, their biomass can be quite high and they occupy important places in marine food webs (for review and literature see Bone 1998). Ascidians are convenient to collect and very useful experimental material; consequently, they are the best known protochordates and as a result the majority of this overview deals with them. Lambert (2005) examines the ecology and natural history of protochordates, which is the first comprehensive review of the field in over 30 years (Millar 1971).

Ascidians are generally sessile with no resemblance to vertebrates, but their larvae have chordate traits, including a notochord, dorsal tubular nerve cord, and pharyngeal gill clefts. They constitute the most numerous class of tunicates. Because they are mostly sessile as adults, through history they have been variously classified as plants or animals (Berrill 1950). Aristotle recognized their animal affinities, and they were assigned to various groups as science progressed and techniques for observation became more acute. Mainly, the colonial forms were considered to be cnidarians that were related to corals (Monniot et al. 1991). Linnaeus in 1767 placed the solitary ascidians in the genus *Ascidia* and described *Ascidia intestinalis*, which now is in the genus *Ciona* and is probably the world's best known ascidian. Lamarck (1816) named the group Tunicata, and Cuvier (1815) added much to our knowledge of their anatomy but, along with many others, classified them with lamellibranch mollusks, an affinity that was endorsed as late as 1905 (Alder and Hancock 1905–1907). Savigny (1816) clearly showed the relationship between solitary and colonial ascidians and also showed that they were not allied with the lamellibranch mollusks. Through most of the remainder of the 19th century there were numerous publications on the structure, larvae, and metamorphosis of ascidians, but no one had pointed out their obvious chordate nature. In 1867, Kowalevsky published the detailed development and structure of the eggs and larvae of *Ciona intestinalis* and *Phallusia mammillata* and pointed out their affinity with the Chordata. This finding electrified Darwin as well as Huxley and other champions of Darwin's *Origin of the Species By Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. A paradigm shift was required to accept that tunicates were chordates, not mollusks, and such a shift required time as many workers who considered them mollusks were replaced by those who agreed with Kowalevsky.

During the 19th century numerous coastal areas were extensively explored and many ascidians described, which resulted in many fine taxonomic publications. As more species were described and details of their anatomy were revealed, it became necessary to erect classification schemes to better enable scientists to categorize taxa into manageable groups.

Foremost in these efforts was the work of Lahille (1886) that proposed three orders based upon the structure of the branchial sac: Aplousobranchia, Phlebobranchia, and Stolidobranchia. Many of us today still prefer this categorization. In 1898, Perrier recommended another scheme based upon the position of the gonads. The Enterogona included Lahille's Aplousobranchia and Phlebobranchia and the Pleurogona included only the Stolidobranchia. Subsequently, many workers adopted Perrier's classification, with Lahille's orders becoming suborders (Berrill 1950). However, this idea implies that the aplousobranchs are more closely related to the phlebobranchs than either group to the stolidobranchs. Until such time as this is verified by molecular or other means, it would be prudent to continue to apply Lahille's orders, especially since they have precedence.

Increasing interest in the structure, development, physiology, and biogeography of ascidians led to several valuable books and major monographs on all aspects of their lives. Herdman (1882, 1886) admirably reviewed the major works of the 19th century. Huus (1937) reviewed many articles that dealt with all aspects of ascidians, including their structure, development, biogeography, and classification. Brien (1948) reviewed structure and reproduction. Berrill (1950) continues to be a widely used classic reference on all aspects of ascidians with a description of the British species; his subsequent book in 1955, *The Origin of Vertebrates*, though still useful contains some ideas that are no longer accepted. The Monniots published a superb overview of the class as part of their account of tropical ascidians (Monniot et al. 1991). *The Biology of Ascidians* (Sawada et al. 2001) with a collection of 69 papers on all aspects of ascidians appeared in 2001.

In addition to these overview accounts, more specialized reviews have emphasized specific attributes of the protochordates. Several aspects of the biology of the group appeared in *Protochordates* (Barrington and Jefferies 1975). Millar (1971) reviewed ecology and Goodbody (1974) reviewed ascidian physiology. Satoh (1994), and later, Jeffery and Swalla (1997) reviewed the extensive literature on development and Whittaker (1997) reviewed cephalochordates in the same volume. A special issue of the *American Zoologist* contained a series of papers on ascidian development in 1982. The recent review by Burighel and Cloney (1997) admirably summarizes ascidian structure and function.

Fertilization and development

Many hemichordates produce a swimming larva similar to an echinoderm larva, one of the similar traits between hemichordates and echinoderms. Enteropneusts are exclusively dioecious, but pterobranch colonies can include both sexes. Hemichordates from both classes show extensive asexual development (Hadfield 1975). Spawning is apparently epidemic and related to tides or temperature (for a review see Hadfield 1975).

Cephalochordates are usually dioecious but may have skewed sex ratios in nature (Kubokawa et al. 2003). They are incapable of budding or asexual development, but larvae may become sexually mature if blocked from metamorphosis (Wickstead 1975). Whittaker (1997) has thoroughly reviewed their development.

In this issue, Bates (2005) discusses environmental signals and reproductive strategies in ascidians, while here I focus on spawning, fertilization, and development. Light is an important cue in ascidian spawning. Some, such as the phlebobranchs *C. intestinalis* (Lambert and Brandt 1967; Whittingham 1967), *Corella parallelogramma* (Huus 1939), *Corella inflata*, and *Corella willmeriana* (Lambert et al. 1981), spawn within a few minutes of light following darkness. Spawning requires a longer light exposure in several stolidobranchs (Rose 1939; West and Lambert 1976; Numakunai and Hoshino 1980; Jeffery 1990). However, another stolidobranch, *Molgula manhattensis*, requires only a short exposure (Whittingham 1967). Interestingly, *C. intestinalis* populations from different regions have dissimilar responses to light. This species from the Atlantic and Pacific US coasts spawns in light following darkness (Lambert and Brandt 1967; Whittingham 1967), but the same species spawns in response to darkness following light in the Mediterranean (Georges 1968; C.C. Lambert, unpublished observation). The target for light reception is included in the gonoducts and dorsal strand of *C. intestinalis* (Reese 1967; Woollacott 1974) and an action spectrum for spawning suggests a cytochrome for the receptor molecule (Lambert and Brandt 1967).

Like many sessile organisms all ascidians are hermaphrodites, although many species are protandrous (Sabbadin et al. 1991). Most solitary ascidians spawn their eggs freely into the sea where fertilization and development take place, but a few species brood within the atrium (Lambert et al. 1995). All colonial forms brood their eggs, either in special brood chambers or in the atrium. Sperm is shed into the sea and some colonial forms can store exogenous sperm for prolonged periods (Bishop and Ryland 1991).

A tadpole larva, which may swim for 12 h to several days, is produced by most free-spawning species; however, larvae from brooded eggs generally swim for only a few minutes or hours (Lambert 1968; Lambert et al. 1995). In this issue, McHenry (2005) reviews swimming of ascidian larvae, and Lambert (2005) and Bates (2005) consider the ecological consequences of a shortened swimming period. Still another reproductive strategy is seen in several *Molgula* species where the spawned egg develops directly into a functional juvenile without going through a swimming larval stage (Berrill 1931; Bates 2005). Tadpole release from the colony appears to be under the control of light (Watanabe and Lambert 1973; Forward et al. 2000.). Light and gravity also influence the behavior of swimming larvae. The review by McHenry (2005) expands on the behavior of larval ascidians.

Ascidian eggs are enclosed within cellular and noncellular vestments. A layer of test cells surrounds the egg and contributes to the larval tunic (Cloney and Hansson 1996; Takamura et al. 1996). These cells are enclosed within a noncellular vitelline coat (termed chorion in older literature). Around the outside of the vitelline coat (VC) is a single layer of follicle cells that function in protein and RNA synthesis (Jeffery 1980), egg flotation (Lambert and Lambert 1978), and egg adhesion (Young et al. 1988; Lambert et al. 1995) in some species. They are required for maturation in *Halocynthia roretzi* (Sakairi and Shirai 1991), fertilization (Hoshi et al. 1981; Hice and Moody 1988; Fuke and Numakunai 1996), and the block to polyspermy in others (Lambert et al. 1997). They also contribute to the block to

self-fertilization in *C. intestinalis* (Marino et al. 1999) and *H. roretzi* (Fuke and Numakunai 1996). This layer of cells is the inner of two follicle cell layers present in the ovarian follicle (Burighel and Cloney 1997). Ascidian eggs have at least two blocks to polyspermy: the follicle cell-generated fast block and a slower electrical block (Lambert et al. 1997). Polyspermy blocks are essential for normal development, as ascidians often live in massive aggregations and the sperm concentration when the eggs are released may be quite high.

Solitary ascidians generally produce a large number of eggs that are around 150 µm in diameter, whereas compound forms usually have only a few but much larger eggs. Forms with external fertilization like most phlebobranchs and stolidobranchs spawn small, rather simple sperm; those with internal fertilization like all aplousobranchs have larger and more complex sperm (Lambert 1982; Martinucci et al. 2001). Sperm from solitary ascidians can swim for extended periods and undergo hypermotility in the vicinity of conspecific eggs (Bolton and Havenhand 1996). In addition, ascidian eggs release factors that increase the motility of sperm and can cause the directed swimming of the sperm to the egg (Miller 1975; Morisawa et al. 2001; Yoshida et al. 2002; Ishikawa et al. 2004).

Sperm of hemichordates, cephalochordates, and appendicularians have a large apical acrosome and a midpiece posterior to the nucleus. During fertilization an acrosomal filament extends and fuses with the egg (for a review see Jamieson 1991). In contrast, the sperm of ascidians and thaliaceans have a much reduced acrosome and lack a classical midpiece. The single mitochondrion is adjacent to the nucleus in the head (Retzius 1904, 1905; Franzén 1976; Villa 1977). During fertilization the mitochondrion slides down the tail by an actin–myosin driven process (Lambert and Lambert 1984) as the sperm passes through the VC (Lambert and Epel 1979; Lambert and Lambert 1983; Lambert 1989; Lambert and Battaglia 1993).

All ascidians are hermaphrodites, but many cannot fertilize their own eggs. For instance *C. intestinalis*, *Ciona savignyi*, and all pyurids such as *Halocynthia* and *Pyura* species are generally self-sterile, whereas many other species including many styelids and most ascidiids are self-fertile, at least in the laboratory. The mechanism of this specificity involves a particular protein in the VC (Fuke and Numakunai 1996, 1999; Marino et al. 1998, 1999; Sawada and Yokosawa 2001). Removal of the VC allows self-fertilization (Byrd and Lambert 2000). The sperm binds to the egg through a sperm surface glycosidase binding to a VC glycoside (Hoshi 1986; Lambert and Koch 1988; Honegger 1992) or through a sperm proteasome binding to extra cellular ubiquitin (Sawada et al. 2002). Fertilization is species-specific as long as the VC is present (Reverberi 1971; Patricolo and Villa 1992), but sperm interaction with follicle cells is not. This can lead to interspecific sperm competition: sperm from *Ascidia sydneiensis* and *Phallusia julinea* interfere with the fertilization of *Phallusia nigra* eggs (Lambert 2000). The block to self-fertilization in both *C. intestinalis* and *C. savignyi* is removed by acid treatment, but this does not allow interspecific hybridization (Byrd and Lambert 2000).

Interspecific fertilization is generally not possible, but it is possible to obtain fertilization between two *Molgula* species

in which one has a tailed larva and the other lacks a tail. In this cross, the molecular switches to anural development have been discovered (Swalla and Jeffery 1990; Swalla et al. 1999).

Following fertilization, the egg experiences multiple pulses in intracellular calcium emanating from the fertilization site (Roegiers et al. 1995; Dumollard et al. 2004), leading to cortical and endoplasmic reticulum reorganization in the zygote (Speksnijder et al. 1995). Conklin (1905a) was the first to note the pigmented plasms present in the zygotes of *Styela canopus* (formerly *Cynthia partita*). He found that the plasms became localized in distinct parts of the tadpole larva and was able to determine the cell lineages involved. Subsequently, pigmented plasms have been seen in eggs of *Boltenia villosa*, *Styela plicata*, and a few other species; however, most ascidian zygotes and embryos lack such markers. Conklin (1905b) went on to confirm, by blastomere deletion studies, that ascidian eggs were strictly mosaic and only right or left half larvae were produced from one of the first two blastomeres, a finding that has been verified by many other studies with various species. This is an intriguing result in view of the tremendous regenerative powers of adult ascidians. Indeed, half embryos can metamorphose into fully functional adults with all structures present (Nakauchi and Takashita 1983). The numerous studies implicating mosaic development and autonomous differentiation in ascidian embryos have led to many investigations of the ability of one part of the embryo to influence other cells. In an early experiment with *S. canopus* embryos, Rose (1939) showed that neural induction was necessary for the formation of the brain. Later studies have uncovered many other cases. The molecular nature of autonomous differentiation and induction in ascidian embryos continues to be a very active field of research that has been extensively reviewed (Satoh 1994, 1998, 2001, 2003; Corbo et al. 2001; Makabe et al. 2001; Nishida 2002). In this issue, Cone and Zeller (2005) review the evolution of developmental gene networks, while Shimeld and Holland (2005) analyze the extensive findings on the molecular biology of cephalochordate development and how these findings impact our concept of the evolution of the chordates.

Ascidian colonies are composed of numerous small zooids embedded in a common tunic or small individual zooids connected by stolons. Coloniality is not related to systematic position. Colonial species are common in two orders: all aplousobranchs and stolidobranchs from the family Styelidae. The only colonial phlebobranchs are *Perophora* species and the poorly understood Plurellidae. Ascidians use multiple asexual methods of budding to produce colonies (reviewed by Nakauchi 1982; Nakauchi and Kawamura 1990; Satoh 1994). Life cycles of zooids in *Botryllus schlosseri* colonies show a regular alternation between budding and death by apoptosis (Lauzon et al. 2002). Colonies grow larger by spreading on the substrate and frequently two colonies grow into each other. They can either fuse or reject each other when they meet. Colony fusion and rejection has fascinated biologists for decades and continues to be an active area of research; in this issue, Rinkevich (2005) surveys the function of the immune system during colony fusion. In many stolidobranchs, such as *Botryllus* species, colony fusion is genetically controlled (Scofield et al. 1982; Watanabe and Taneda 1982; for reviews see Satoh 1994), but colonies of the aplousobranch *Diplosoma listerianum* fuse indiscriminately and the resulting colony is a chimera containing zooids of several genotypes (Bishop and Sommerfeldt 1999).

Structure and function

Structure and function are inextricably linked and have been extensively reviewed (Goodbody 1974; Kott 1989a; Burighel and Cloney 1997). In this issue, Lacalli (2005) reviews protochordate body plans and the evolution of larvae, while Cameron (2005) reviews the morphological phylogeny of the hemichordates. In addition, Ruppert (2005) reviews the relationship and evolution of structural similarities between the protochordate phyla.

Hormones are chemical signals operating to convey messages within the organism. Most were originally discovered in vertebrates or insects, but several are also found in protochordates and are important in evolutionary and functional issues. The review by Sherwood et al. (2005) on the endocrinology of protochordates covers the known hormones and their receptors, as well as the evolution of the endocrine system.

Protochordates have evolved nervous systems quite different from most protostomes and the other deuterostomes. Certain similarities exist between the protochordate and vertebrate nervous systems, and these are crucial to understanding evolutionary relationships. Because the central and peripheral systems of the ascidian larva have the simplest structure in the chordate lineage, being composed of only a few hundred cells (Nicol and Meinertzhagen 1991), and because of their invariant cleavage pattern (Meinertzhagen and Okamura 2001), development of this system is under intense scrutiny (Meinertzhagen et al. 2000). In this issue, Meinertzhagen (2005) reviews the development, structure, and function of the larval ascidian nervous system, and Mackie and Burighel (2005) review the adult nervous system. Cephalochordates exhibit a very much reduced degree of cephalization compared with vertebrates. Consequently, the structure and function of their nervous system are proving crucial to understanding protochordate relationships (see review by Wicht and Lacalli 2005).

These reviews show that while we have learned a great deal about protochordates, there is still much more work to be done to understand this key evolutionary group of organisms. "The study of animals is always the study of unresolved problems, and of no group is this more true than the protochordates" (Barrington 1965). We hope that these reviews inspire renewed efforts and serve to focus research into unresolved questions.

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